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BIOCHEMICAL JOURNAL

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THE

BIOCHEMICAL JOURNAL

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BY

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OBITUARY NOTICES

HAROLD ACKROYD

Harold Ackroyd was the son of Mr Edward Ackroyd, for many years chairman of the Southport and Cheshire Lines Extension Railway. His schools were Mintholme College, Southport, and Shrewsbury. He joined Gonville and Caius College, Cambridge, and took his B.A. in 1899. In the following year he attended the lectures and practical classes as then arranged for physiology in Part II of the Natural Sciences Tripos, though, from the first, he had no intention of entering for the examination. In the work of the chemical section I remember how good for his teacher was the critical attitude he displayed towards the methods used in the practical classes. His were criticisms not verbally expressed but set down in his note book in the form of figures. While others were for the most part content to employ a method "as directed" Ackroyd was apt to test first the limits of its accuracy. Then, as later, and always, he was impatient of compromise, and had no use for the second best, in laboratory methods or anything else; but once at work he spared himself no pains to reach the highest grade of accuracy possible under the circumstances.

He went to Guy's Hospital for his clinical training and took his medical degrees in due course. Later he was House-Surgeon at Queen's Hospital, Birmingham, and then worked for a time at the David Lewis Northern Hospital, Liverpool. He possessed ample private means and never attempted ordinary medical practice.

He hesitated a good deal before deciding to devote himself entirely to scientific research though so well qualified to do it. He was fond of travel and had a passion for the sea. While undecided about his career he indulged these tastes freely, going for long voyages in slow boats and exploring Europe pretty thoroughly.

After his marriage he decided to settle down to biochemical research at Cambridge. He went therefore to reside at Royston, and attached himself, first to the Department of Pharmacology, and afterwards to the School of Agriculture where he accepted a post in the newly founded Institute for the study of Animal Nutrition. He had previously held for some time a British

Medical Association Scholarship. He concerned himself chiefly with the subject of purine metabolism. In 1910 he published a paper showing that when urates are perfused through the liver of the dog they are, partly at least, converted into allantoin, without any formation of urea. Allantoin itself was shown to undergo no change[1]. In a later paper[2] he showed that in the case of rabbit's liver the conversion of uric acid into allantoin is complete, the latter proving once more to be an end product. He demonstrated[3], after a careful analysis of various foodstuffs, that the small quantity of allantoin found in human urine may wholly arise from the diet, even when it consists of milk and vegetables only. Ackroyd's work on these lines gave strong support therefore to the views of Wiechowski, then under discussion, by showing that allantoin, while the end product of purine metabolism in animals, is probably not produced at all in the human body.

For a long time while at the School of Agriculture he engaged most patiently in a laborious study of the nitrogenous constituents of root-crops. The results unfortunately have never been published. A few months before the war I proposed to him some conjoint work upon possible precursors of purines in the body. He began the work, and a preliminary paper[4] was published in December, 1916—a paper which, unhappily, Ackroyd never saw.

In February, 1915, he was commissioned as temporary lieutenant in the R.A.M.C., being afterwards promoted captain. He became attached to the R. Berks Regiment and went to France in August, 1915. He was slightly wounded on July 31st, 1917, but remaining on duty was killed in action on August 11th. Before his death he received notification that he had been awarded the Military Cross for conspicuous bravery as a battalion doctor, but the crowning honour of the V.C. was a reward for his final sacrifice. It, together with the M.C., were received from the hands of the King by his widow and one of his small sons. The London *Gazette* thus describes the conduct for which he was granted the V.C.:

"Utterly regardless of danger he worked continuously for many hours up and down and in front of the line, tending the wounded and saving the lives of officers and men. In so doing he had to move across the open under heavy machine gun, rifle, and shell fire. He carried a wounded officer to a place of safety under very heavy fire. On another occasion he went some way in front of our advanced line and brought in a wounded man under continuous sniping and machine gun fire. His heroism was the means of saving many lives, and provided a magnificent example of courage, cheerfulness, and determination to the fighting men in whose midst he was carrying out his splendid work. This gallant officer has since been killed in action."

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[1] This *Journal*, 1910, 5, 217.

[2] *Ib.* 442.

[3] *Ib.* 400.

[4] This *Journal*, 1916, 10, 551.

To one who knew him best as a colleague patiently engaged in the details of laboratory work these eloquent words bring a strange sense of contrast. Ackroyd however brought courage to every task and did everything well.

F. G. Hopkins.

SAMUEL GEOFFREY SCOTT.

S. G. Scott came up to Magdalen College, Oxford, in 1893, and took the Final Honour School of Physiology in 1897. During his undergraduate career he was taught Histology by Dr (now Professor) Gustav Mann, and became his devoted disciple, not only in methods of technique, but especially in his regard for Histology as a subject of Micro-Chemistry rather than of minute anatomy. This early enthusiasm persisted and increased till it became the sole aim of his scientific life and thought.

After completing his medical studies in London, Scott became demonstrator of Pathology at Leeds in 1903, at first under Dr Trevelyan, and subsequently under Prof. A. S. Grünbaum. From the nature of his teaching he became drawn to pathological research, and he published several papers in the *Journal of Pathology*, the *Lancet* and elsewhere, dealing with the microscopic anatomy of diseased organs. These include "A Case of Sub-Acute Atrophy of the Liver" (1905), "Obstruction Atrophy of the Pancreas" (1907), and other papers on Multiple Neurofibromatosis, Infantile Splenic Anaemia, Leukaemia, and Aplastic Anaemia, some of these being produced in conjunction with Dr W. H. M. Telling.

In 1908 Scott returned to Oxford as lecturer and demonstrator in Histology, and here he at once settled down to the work for which he had so rare and exceptional an aptitude. On the technical side of Histology he was a master, and his microscopical preparations were perfect. Where his profession was concerned he would admit of nothing second rate, if it could be avoided, and if an innocent undergraduate consulted him as to the microscope he should invest in, he was shown a programme of stand, objectives and eye-pieces costing over £100. Yet such was Scott's persuasiveness and enthusiasm that quite a number of the better-endowed undergraduates followed his advice implicitly. Scott practically lived in the laboratory, as he did not trouble about such mundane matters as lunch, and very rarely took a holiday. He was always ready to spend unlimited time in giving advice to pupils and colleagues, and to pour out his ideas and theories on the subject so dear to his heart, viz., the chemistry of staining. Of almost every dye he knew not only its staining properties, but its chemical constitution, and nothing pleased him better than to cover blackboard or paper with formulae indicating the probable linkage whereby the stain attached itself to the object stained, and the chemical transformations it underwent during bleaching, change of reaction, and so on. He was very careful and cautious in committing himself in print, and so had accumulated a number of not quite complete investigations, which now, alas, will never be finished off and see the light. His chief published work on micro-chemistry includes two important papers on Romanowsky Staining for Blood Cells (*Journ. of Path.*, Vol. 16, 1911, and *Folia Haematologica*, Vol. 12, 1911), which he produced in conjunction with two of his pupils, T. O. Thompson and J. L. Hydrick.

On the outbreak of the war Scott took up a temporary appointment as house surgeon at the Radcliffe Infirmary, Oxford, and later, finding it was possible to obtain a substitute for his teaching work, he thought it his duty to volunteer for a commission in the R.A.M.C. In the early summer of 1917 he went to France, and later on to Italy. Here he succumbed in a few days to an attack of pneumonia. His friends and colleagues can never forget his genial presence, his unfailing good temper and his intense devotion to his work. They offer their deepest sympathy to his widow.

H. M. Vernon.

I. OBSERVATIONS ON THE PHOSPHOTUNGSTATES OF CERTAIN BASES AND AMINO-ACIDS.

By JACK CECIL DRUMMOND.

From the Biochemical Laboratory of the Cancer Hospital Research Institute, London.

(*Received July 18th, 1917.*)

INTRODUCTORY.

In spite of the extensive use which is made of phosphotungstic acid as a precipitant for organic bases, comparatively few investigators have studied the individual salts of the acid or made any attempt to define the most suitable conditions for their preparation.

The observations recorded in this communication were made during an attempt to separate complex mixtures of bases and amino-acids by a process depending upon the relative solubilities of their phosphotungstates in various solvents.

Unfortunately the study is to a large extent incomplete, and, since there does not appear to be any likelihood of the opportunity arising whereby the work may be completed, it has been decided to publish the few results which have been obtained in the hope that they may either be of some slight practical utility, or that they may suggest more extensive investigations along similar lines.

Phosphotungstic acid was introduced as a precipitant for organic bases by Drechsel, and since that introduction its use for that purpose has become more and more extensive. References to the use of the acid are scattered so widely apart throughout the literature that it is difficult to review them at all completely. With regard to the acid itself, its preparation is described by Drechsel [1887], and also by Winterstein [1898]. The two methods are practically identical in detail and give a yield of from two to three parts of the acid from four parts of sodium tungstate and one part of sodium phosphate. The acid prepared by this method is usually somewhat impure and it is necessary in such cases to subject it to a process of purification by ether treatment (Winterstein). Reference to the literature shows that a large series of phosphotungstic acids have been described, mainly by Kehrmann and his co-workers [1887, 1888, 1892, etc.]. These acids possess different proportions of P_2O_5 and WO_3. Apparently the acid prepared by the process referred to above, which is that sold commercially, forms salts of the type

$$R_3, H_3PO_4, 12WO_3, xH_2O.$$

(R being a monovalent base.)

Gulewitsch [1899] prepared and submitted to analysis pure preparations of arginine phosphotungstate and found that his analytical data satisfied the formula $(C_6H_{14}O_2N_4)_3, 2H_3PO_4, 24WO_3, 10H_2O$.

Similarly Barber [1906] in an investigation of the phosphotungstates of the simpler amino-acids gives a similar formula for:

glycine phosphotungstate	$(C_2H_5O_2N)_3, H_3PO_4, 12WO_3$,
alanine ,,	$(C_3H_7O_2N)_3, H_3PO_4, 12WO_3$,
glutaminic acid ,,	$(C_5H_9O_4N)_3, 2H_3PO_4, 24WO_3$.

In the case of the salts of aspartic acid and asparagine, however, he suggests formulae of a different type:

asparagine phosphotungstate	$(C_4H_8O_3N_2)_5, 2H_3PO_4, 22WO_3$,
aspartic acid ,,	$(C_4H_7O_4N)_4, 2H_3PO_4, 20\text{–}22WO_3$.

Wechsler [1911] confirmed the formula given by Gulewitsch for arginine phosphotungstate and found that a similar one, $(C_6H_9O_2N_3)_3, 2H_3PO_4, 24WO_3$, satisfied the analytical data for the histidine salt.

A considerable number of salts of phosphotungstic acid with various bases and amino-acids have been prepared during the course of this inquiry and in every preparation submitted to analysis the figures have agreed with the composition indicated by the formula:

$$R_3, H_3PO_4, 12WO_3, xH_2O. \quad (R = \text{monovalent base.})$$

With regard to the studies that have been made of the salts themselves there is little to review. Generally speaking they have been concerned with determinations and comparisons of the relative precipitability of various substances by the acid. Gulewitsch [1899] made a careful investigation of arginine phosphotungstate, and determined the conditions for its optimum precipitability. He pointed out the advantage of utilising a slight excess of the precipitant and of conducting the operation in a medium of 5 % sulphuric acid.

Barber [1906] investigated the phosphotungstates of several of the simpler amino-acids, and recorded their probable constitution, relative precipitability and their solubilities in water, alcohol and 50 % alcohol.

Levene and Beatty [1906] also studied the precipitability of several simple amino-acids.

Other investigations of a similar nature are most difficult to collate since they are scattered so widely throughout the mass of literature dealing with the individual bases themselves. The few that have been traced will be considered when the particular compound to which they refer is being discussed.

EXPERIMENTAL.

1. *Conditions under which maximum precipitation is obtained.*

From a consideration of first principles it is recognised that many factors will influence the precipitability of a substance. In the present case the most important of these factors are temperature, the concentration of base, and the degree of acidity of the medium in which the reaction is carried out.

The study of arginine phosphotungstate made by Gulewitsch [1899] included an experimental investigation of the conditions necessary for the optimum precipitation of that compound from a 0·02 % solution of arginine nitrate.

The results of his investigation demonstrated the advisability of using a slight excess of the precipitant and of operating in a medium of 5 % sulphuric acid. When the precipitation occurred in 2·5 or 7 % sulphuric acid, or when too great an excess of the phosphotungstic acid was used precipitation was either incomplete or the precipitate tended to redissolve.

The determinations of precipitability recorded in this paper were made by adopting a technique similar to that employed by Gulewitsch.

A series of numbered, stoppered, 50 cc. volumetric flasks was taken and into each was introduced a known volume of a dilute solution of the base under examination. A 20 % solution of sulphuric acid was then run in to each flask in an amount sufficient to render the contents of the flask of the required degree of acidity when made up to 50 cc. Distilled water was then added in an amount just sufficient to permit the further addition of the required amount of a 30 % solution of phosphotungstic acid in order to bring the volume of liquid in each flask up to 50 cc.

The flasks were then stoppered and shaken vigorously for half a minute, after which they were allowed to stand at room temperature (19°) for 24 hours with occasional agitation.

The contents of the flasks were filtered and determinations of unprecipitated nitrogen made upon aliquot portions of the filtrates.

The following tables indicate the findings in the study of the precipitation of betaïne and histidine from solutions of their salts.

Table I.

Precipitation of histidine from a dilute solution of histidine hydrochloride by phosphotungstic acid under varying conditions. Figures represent mg. nitrogen unprecipitated. (*Temperature* 19°.)

Strength of sulphuric acid present	cc. 30 % phosphotungstic acid solution						
%	0		5	10	15	20	30
0	11·58	—	—	—	5·21	6·00	6·30
2	—	—	—	—	3·99	—	—
3·5	—	—	—	—	3·90	—	—
5	—	7·96	5·25	3·60	3·65	3·50	4·90
7	—	7·96	5·25	4·05	4·00	3·60	5·22
9	—	—	—	—	4·37	—	—
11	—	—	—	—	4·57	—	—
18	—	—	—	—	5·20	—	6·33

Table II.

Precipitation of betaïne from a dilute solution of betaïne hydrochloride by phosphotungstic acid under varying conditions. (*Temperature* 19°.)

Strength of sulphuric acid	cc. 30 % phosphotungstic acid solution					
%	0	5	8	10	20	30
0	3·07	—	—	2·05	—	—
3·5	—	—	—	1·54	—	2·07
5	—	—	1·77	1·43	1·46	1·90
7	—	2·17	1·83	1·47	1·54	1·96
9	—	—	—	1·54	—	—
18	—	2·24	—	1·89	—	—

From a consideration of these figures it will be recognised that the conclusions are similar to those made for arginine by Gulewitsch, in that the most satisfactory precipitation of these two bases occurs when a slight excess of the reagent is used and when the operation is carried out in 5 % sulphuric acid. Gulewitsch recorded the different appearances of the precipitated arginine phosphotungstate obtained under varying conditions, for the beneficial influence of the slight excess of phosphotungstic acid is not as readily observed from the tables as it is from a study of the precipitates themselves. Under conditions such as are indicated above the precipitates tend to become more distinctly crystalline and are therefore more readily filterable.

A convenient form in which to use the phosphotungstic acid is that of a 25–30 % aqueous solution. Many samples of the acid recently purchased [1916] have been somewhat impure, and it is wise never to employ an acid, which fails to dissolve in water to a clear solution, until it has been purified by the ether process sufficiently to pass this test.

Demjanowski [1912] did not find any appreciable difference in the precipitability of creatinine, carnosine and methylguanidine when he employed 2·5 % and 25 % solutions of the reagent, so apparently the strength of the latter is a comparatively unimportant matter.

Re-solution of the precipitate upon adding an excess of the precipitating acid does not always occur. Many phosphotungstates are extremely readily soluble in such excess, whilst others are not so influenced. The condition of the precipitate itself is governed by several factors. Upon standing amorphous precipitates often become slowly crystalline, whilst in many cases slow precipitation from a very dilute solution of the base results in the production of a crystalline precipitate direct.

A modification of the latter process is valuable for obtaining crystals of a relatively large size, if such are desired for any purpose.

The crystalline form of phosphotungstic acid precipitates when derived from the amorphous forms is usually that of masses of thin needles or of short rhombohedral prisms. When however the crystals are allowed to form slowly, as described above, by precipitation from very dilute solution or when the precipitates are slowly recrystallised from suitable solvents it is sometimes possible to obtain specimens which are characteristic of the particular salt.

Unfortunately the number which yield such characteristic crystals is small, and it is only very occasionally that the crystalline form of the salt is any guide to the recognition of the parent base. Certain of the phosphotungstates examined during the course of this investigation possessed colour and in certain cases this is helpful for purposes of identification.

2. *Preparation of the purified phosphotungstates.*

The phosphotungstates were in all cases prepared by the addition of a very slight excess of 30 % phosphotungstic acid to a solution of the base or its salt in 5 % sulphuric acid. After standing for 24 hours the precipitates were filtered off and well washed with 5 % sulphuric acid. The products thus obtained were submitted to an initial purification by recrystallisation from hot water and the crystalline salts dried over sulphuric acid in a darkened and evacuated desiccator. The storage of the pure products should be carried out in the absence of light since many of them tend to assume a blue or violet colour if exposed to photochemical influences.

When prepared by such a process the phosphotungstates are in the large majority of cases well defined, crystalline products. A few of them are coloured but the greater number are colourless. The crystalline form of the salts varies with the conditions of crystallisation. If this is rapid the crystals are usually thrown out of solution in thick felted masses of needles. On the contrary slow crystallisation produces well formed crystals which are in some cases sufficiently large to be examined with a pocket-lens.

The choice of a solvent for recrystallisation of the phosphotungstates can be made from water, ethyl alcohol, methyl alcohol, acetone or mixtures of two or more of these.

The experience gained during the present research has led to the adoption of water or 50 % ethyl alcohol as being the most suitable solvents for the purpose. Even when recrystallised from the same solvent the crystalline form of an individual phosphotungstate may on different occasions exhibit differences, so that the identification of any particular salt by this means is a somewhat untrustworthy process.

3. *Analysis and composition of the purified phosphotungstates.*

The purified salts obtained by the process just described were in a number of cases submitted to analysis. Moisture was determined by the usual process of drying to constant weight at 110°. Nitrogen was estimated by Kjeldahl. In some cases determinations of tungsten oxide were carried out. Various procedures for conducting the latter analysis were tested, the best results being obtained by the method of Barber [1906], also used with good results by Wechsler [1911]. Many of the phosphotungstates were submitted to analysis after more than one recrystallisation and it was generally found that their composition remained unaffected by the purification. Apparently some compounds are so affected, for Barber [1906] states that upon recrystallisation of glutaminic acid phosphotungstate isomorphous mixtures of the salt and the free acid are obtained.

4. *Determinations of Solubility.*

Determinations of the solubility of the individual phosphotungstates in various solvents were carried out where the amount of material permitted. When this was not possible qualitative tests of this nature were made. The solubilities were estimated by determining the weight of salt dissolved in a known volume of solvent after they had remained in contact for 24 hours at room temperature with occasional agitation.

5. *Properties of the Individual Phosphotungstates.*

(*a*) *Amino-acids.*

Glycine phosphotungstate, was studied by Barber [1906], who prepared it by the addition of 50 % phosphotungstic acid solution to a saturated solution of the amino-acid. He describes it as crystallising in microscopic whetstone-shaped crystals. On attempting to dissolve it in warm water he found that tungstic acid was thrown out of solution.

Preparations dried at 105° gave upon analysis figures which agreed with the formula, $(C_2H_5ON_2)_3, H_3PO_4, 12WO_3$. It normally occurs with water of crystallisation, approximately 5–6 molecules being present. Recrystallisation from hot alcohol results in glycine being deposited. Levene and Beatty [1906] record the precipitability of glycine by phosphotungstic acid.

Alanine phosphotungstate was described by Barber [1906] as crystallising in microscopic twin needles. It retains its composition after recrystallisation. Its analysis agrees with the formula $(C_3H_7O_2N)_3 . H_3PO_4, 12WO_3, 4—5H_2O$. Levene and Beatty [1906] have studied its precipitability.

Leucine phosphotungstate. Barber [1906] could not prepare leucine phosphotungstate, and a similar failure was reported by Schulze and Winterstein [1901]. Levene and Beatty [1906], however, describe it as an oily product soluble in a small excess of the reagent.

Aspartic acid phosphotungstate. Barber [1906] states that it occurs in the form of stubby microscopic octahedra containing 24 molecules of water of crystallisation, and varying in composition, with the formula $(C_4H_7O_4N)_4 . 2H_3PO_4, 20—22WO_3$. Its composition usually agrees most satisfactorily with $22WO_3$ and it is unchanged by recrystallisation. Levene and Beatty [1906] studied the precipitability of aspartic acid by phosphotungstic acid.

Glutaminic acid phosphotungstate is described by Barber [1906] as a flocculent mass which slowly becomes crystalline. Its composition as indicated by the analytical figures indicates that it readily forms isomorphous mixtures with the free acid. Levene and Beatty [1906] describe it as slightly soluble in excess and they record its precipitability.

Cystine phosphotungstate was described by Winterstein [1901] who obtained a crystalline product. Plimmer [1913] has attempted to utilise the fact that cystine is completely precipitated from all but very dilute solutions by phosphotungstic acid as a basis for a method of separating cystine and tyrosine.

Cystine is readily precipitated from solution by means of phosphotungstic acid. The precipitate is usually of a pale cream colour and if thrown out of dilute solutions may be crystalline.

Upon recrystallisation from hot water, in which it is readily soluble, it separates out in very small rhombohedral tables of a pale sulphur yellow colour (Photograph 4). It does not alter in composition upon recrystallisation.

It usually contains 1·25 % water, equivalent to 4 – 5 molecules of water of crystallisation.

0·2041 g.; 17·6 cc. 0·01N NH_3 = 1·2 % N

0·2046 g.; 18·0 ,, ,, ,, = 1·2 % N.

Calculated for $(C_6H_{12}O_4N_2S_2)_3, 2H_3PO_4, 24WO_3, 4—5H_2O$ = 1·28 % N.

It is very soluble in alcohol, methyl alcohol and acetone.

Phenylalanine phosphotungstate was described in the first place by Schulze and Winterstein [1901] as a pale yellow oil which becomes crystalline upon standing. They state that it is not very soluble in cold water and that it is not precipitated from dilute solutions of the amino-acid. Levene and Beatty [1906] record a similar description of the compound. For this investigation it was prepared from a strong solution of phenylalanine in 5 % sulphuric acid. It separates initially as a lemon yellow oil, sometimes possessing characteristic odour. Upon standing the oil becomes crystalline, forming masses of thin plates and needles. It is readily recrystallised from hot water and after separating as an oil again soon crystallises. The crystals are short rhombohedral prisms which on account of their colour are fairly characteristic. It contains approximately four molecules of water of crystallisation.

Upon analysis it gave the following figures:

0·2071 g.; 16·0 cc. 0·01 N NH_3 = 1·1 % N

0·2065 g.; 15·5 ,, ,, ,, = 1·1 % N

0·1032 g.; 0·0855 g. WO_3 = 83·0 % WO_3.

Calculated for $(C_9H_{11}O_2N)_3, H_3PO_4, 12WO_3, 4H_2O$, N = 1·22 %, WO_3 80·9 %.

Its composition is constant after repeated recrystallisation.

Tyrosine phosphotungstate was not obtained by Schulze and Winterstein [1901], nor by Barber [1906], moreover all attempts made during the course of this work to isolate the compound failed.

Histidine phosphotungstate. Wechsler [1911] has made an analysis of histidine phosphotungstate and considers that it is represented by the formula $(C_6H_9O_2N_3)_3, 2H_3PO_4, 24WO_3$.

It is readily prepared as a white microcrystalline powder. Upon precipitation from very dilute solutions of the base or by recrystallisation from water or dilute alcohol, it may be obtained in comparatively large, clear, well-formed crystals consisting of rhombohedral prisms. (Photograph 3.)

Wechsler describes the substance as anhydrous. Specimens prepared during this investigation, however, gave on analysis indications of the presence of five molecules of water of crystallisation. Its composition remains unaltered with purification.

0·2083 g.; 3.45 cc. 0·1 *N* NH_3 = 2·3 % N
0·2059 g.; 3·20 cc. „ „ = 2·2 % N
0·1959 g.; 0·1708 g. WO_3 = 87·2 % WO_3
0·2170 g.; 0·1885 g. „ = 86·9 % WO_3.

Wechsler gives the following analysis of his product.

2·00 2·08 2·06 % N
86·4 89·8 — % WO_3.

Calculated for $(C_6H_9O_2N_3)_3, 2H_3PO_4.24WO_3$: N = 2·02 %, WO_3 = 89·3 %.
Calculated for $(C_6H_9O_2N_3)_3, 2H_3PO_4, 24WO_3.5H_2O$. N = 1·98 %. WO_3 = 87·2 %.

The salt is very soluble in acetone and methyl alcohol but comparatively insoluble in water and alcohol.

Arginine phosphotungstate. Gulewitsch [1899] made a fairly complete study of this compound. Reference has already been made to his observations upon the conditions influencing its precipitation.

His preparations were submitted to analysis and gave the following data:

N 2·84 2·68 %
C 3·48 3·44 %
H 0·91 0·74 %.

From these figures he considered the substance was to be represented by

$$(C_6H_{14}O_2N_4)_3, 2H_3PO_4, 24WO_3, 10H_2O$$

which requires C = 3·46, N = 2·6, H = 0·65.

Wechsler [1911] agrees with this formula since his preparations contained 2·64 % and 2·70 % nitrogen.

Several preparations made during the course of this work were analysed, the following results being typical of those obtained.

0·2025 g.; 3·20 cc. 0·1 *N* NH_3 = 2·2 % N
0·2096 g.; 4·15 cc. „ „ = 2·8 % N
0·2288 g.; 0·2014 g. WO_3 = 88·0 % WO_3
0·2394 g.; 0·2104 g. „ = 87·9 % WO_3.

The substance contained water of crystallisation equivalent to 6—10 molecules.

Calculated for $(C_6H_{14}O_2N_4)_3, 2H_3PO_4, 24WO_3, 6H_2O$, N = 2·63 %, WO_3 = 87·2%.

The phosphotungstate prepared in the usual manner is a white amorphous powder. It may be readily obtained in the form of rhombohedral prisms of a comparatively large size by recrystallisation from water or dilute alcohol (Photograph 5). The salt tends to redissolve in the presence of a great excess of the reagent, a point also noted by Gulewitsch.

Lysine phosphotungstate is precipitated from great dilutions of the salts of the amino-acid as a white amorphous powder. It may be obtained in a crystalline condition by recrystallisation from dilute alcohol and usually separates out in large clusters of very fine needle-shaped crystals. It retains its composition although subjected to many recrystallisations and is anhydrous. Upon analysis it yields figures which satisfy the formula

$(C_6H_{14}O_2N_2)_3, 2H_3PO_4, 24WO_3$.

0·2089 g.; 18·0 cc. 0·01 *N* NH_3 = 1·2 % N
0·2094 g.; 19·0 cc. „ „ = 1·3 % N.
Required for above formula 1·35 % N.

No other amino-acid phosphotungstates were investigated in this research. The solubilities of those whose other properties have been described are given in the table of solubilities at the end of the paper.

(*b*) *Amines.*

The basic nature possessed by representatives of this class of compound is responsible for the formation of a series of well-defined salts with phosphotungstic acid. Generally speaking they are precipitated from very dilute solutions of the parent base as heavy insoluble microcrystalline powders. One or two of them possess colour which renders their identification possible.

Trimethylamine phosphotungstate is precipitated from exceedingly dilute solutions of the base as a heavy white amorphous powder. It may be obtained in the form of microscopic octahedra by recrystallisation from water. Its composition agrees with the formula $((CH_3)_3N)_3, H_3PO_4, 12WO_3$.

Analysis: 0·2011 g.; 18·9 cc. 0·01 *N* NH_3 = 1·3 % N.

Calculated for above formula = 1·37 % N.

It is readily soluble in acetone but not appreciably soluble in the other solvents examined.

Isobutylamine phosphotungstate separates out as a heavy white flocculent precipitate from solutions of the base upon the addition of phosphotungstic acid. It is very soluble in acetone, but not greatly so in water or ethyl alcohol. It crystallises in large clear rhombohedral prisms.

Isoamylamine phosphotungstate is closely similar to the preceding compound in its crystalline appearance and solubilities.

Tri-isobutylamine, Tripropylamine and Tri-isoamylamine phosphotungstates are closely similar to each other. They are all precipitated from great dilution as heavy white, amorphous precipitates. They crystallise in microcrystalline rhombohedral prisms and tablets, and occasionally in fern-like aggregates closely resembling those assumed by ammonium chloride.

Their solubilities are similar, all being exceedingly soluble in acetone or dilute acetone, practically insoluble in water and ethyl alcohol and slightly soluble in methyl alcohol. The composition of two of these salts was investigated.

Tri-isobutylamine phosphotungstate.

0·4699 g. anhydrous salt; 4·4 cc. 0·1 *N* NH_3 = 1·3 % N.
Required for $(C_{12}H_{27}N)_3, H_3PO_4, 12WO_3$ = 1·23 % N.

Tri-isoamylamine phosphotungstate.

0·5832 g. anhydrous salt; 3·6 cc. 0·1 *N* NH_3 = 1·4 % N.
Required for $(C_8H_{11}N)_3, H_3PO_4, 12WO_3$ = 1·3 % N.

Putrescine and Cadaverine phosphotungstates. Putrescine and cadaverine are both precipitated from very dilute solution by phosphotungstic acid. The resulting salts are heavy white amorphous powders which can be obtained in a microcrystalline condition by recrystallisation from water or alcohol. The crystals are not characteristic, being as is the case with the majority of these salts, rhombohedral prisms.

They exhibit similar solubilities to one another. Acetone or dilute acetone readily dissolves them, but in water they are practically insoluble. Ethyl and methyl alcohol will take up a fair amount of the salts.

Phenylethylamine phosphotungstate. This compound closely resembles the related phenylalanine phosphotungstate in many of its properties. It is not precipitated from dilute solutions of the base except on long standing. It separates out initially as a pale sulphur yellow oil which slowly becomes crystalline. On recrystallisation from water the coloured oil once again separates but the crystals which subsequently form are fairly large and well-formed. They usually appear as pale yellow prisms and tablets, closely resembling the salt of phenylalanine.

The analysis of the purified anhydrous salt gave the data:

0·3517 g.; 3·6 cc. 0·1 N NH_3 = 1·4 % N.
Required for $(C_8H_{11}N)_3, H_3PO_4, 12WO_3$ = 1·3 % N.

Its composition is unaltered by repeated purification. Its solubilities are given in the table on p. 22.

p-*Hydroxyphenylethylamine phosphotungstate* is not precipitated from dilute solutions except on long standing. From concentrated solutions it is thrown out as a straw coloured microcrystalline powder, consisting of flat cubical plates. When deposition occurs slowly or when recrystallisation of the product is carried out with care the compound is readily obtained in the form of large orange-red octahedra, the colour of which resembles that of potassium dichromate.

The salt obtained by these methods is anhydrous and its composition corresponds to the formula $(C_8H_{11}ON)_3, H_3PO_4, 12WO_3$.

0·2190 g.; 2·05 cc. 0·1 N NH_3 = 1·3 % N.
Required for above formula = 1·28 % N.

It is not very soluble in cold water but readily in hot. It is fairly soluble in ethyl alcohol and very soluble in acetone. The crystalline form and colour of this salt are of help in its identification.

β-Iminazolyl-ethylamine phosphotungstate. This compound is precipitated from great dilutions as is the corresponding amino-acid compound. It separates out as a white microcrystalline powder which often assumes a beautifully crystalline form upon standing. These crystals may also be obtained by recrystallisation from water or dilute alcohol, the salt separating from these solvents in the form of relatively large clear rhombohedra and prisms. Its composition agrees with the formula $(C_5H_9N_3)_3, 2H_3PO_4, 24WO_3$. Its solubilities and other properties are very similar to those of histidine phosphotungstate.

Indolethylamine phosphotungstate. This salt is precipitated from very dilute solutions of the base as a terra cotta coloured amorphous powder. Upon standing this undergoes change and becomes crystalline forming large aggregates of needles possessed of a deep brownish purple colour. Upon recrystallisation the amorphous form usually separates out first, the transition to the crystalline form occurring sooner or later upon standing. The microscopical appearance of the crystals of the salt is characteristic.

Upon analysis the following figures for the determination of nitrogen were obtained.

0·1771 g.; 3·5 cc. 0·1 N NH_3 = 2·8 % N.
Required for $(C_{10}H_{12}N_2)_3, H_3PO_4, 12WO_3$ = 2·5 % N.

The compound is very soluble in acetone and in diluted acetone, and fairly soluble in ethyl and methyl alcohol but is only very slightly soluble in cold water.

(c) *Purine Bases.*

The purine bases, generally speaking, are precipitated from very dilute solutions by phosphotungstic acid and the resulting salts are peculiar in being very slightly soluble, even in acetone. They crystallise in cubical plates and may in some cases be recognised by this microscopical appearance. Determinations of nitrogen by the Kjeldahl process do not yield accurate results since the purine nitrogen is not quantitatively accounted for by that method.

Adenine phosphotungstate is usually precipitated as a pale cream coloured powder. It is readily obtained in the form of clear sulphur-yellow cubical, or whetstone-shaped, crystals by careful recrystallisation from dilute alcohol or acetone (Photograph 2). It is very insoluble in water, and ethyl and methyl alcohols and only very slightly soluble in acetone. Funk [1915] has isolated adenine from the acetone-insoluble fraction of the phosphotungstates from autolysed yeast.

Analysis:

0·1050 g.; 0·0899 g. WO_3; 85·6 % WO_3.
Required for $(C_5H_5N_5)_3, H_3PO_4, 12WO_3$; 86·0 % WO_3.

Guanine phosphotungstate is precipitated from extremely dilute solutions of guanine in the form of a buff coloured amorphous precipitate. Wechsler [1911] states that it possesses a brick red colour but the specimens prepared from pure guanine during this investigation were much paler in colour than he describes. Recrystallised from water or dilute alcohol it forms bunches of straw coloured, needle-shaped crystals or small cubical plates. Its properties are closely similar to those of the corresponding adenine salt.

Hypoxanthine phosphotungstate is precipitated as a pale cream coloured amorphous precipitate. It crystallises in small cubical plates possessing a light straw colour. Its solubilities are very similar to those of adenine and guanine.

Xanthine phosphotungstate is very similar in its properties and its crystalline appearance to the hypoxanthine salt.

Uric acid phosphotungstate. The phosphotungstate of uric acid is referred to by Hammarsten [1909, p. 672] as a brick red coloured powder.

It is prepared by precipitation of a solution of sodium urate by phosphotungstic acid and forms a deep chocolate brown powder. It cannot be recrystallised from water or alcohol owing to formation of insoluble uric acid. If prepared from hot sodium urate solutions it may be obtained in a crystalline condition, the crystals being cubical plates and of a deep red brown colour.

(*d*) *Betaïnes and other Bases.*

The betaïnes form well defined crystalline salts with phosphotungstic acid as also do many of the naturally occurring bases. The representatives of both classes are numerous and only a few which were readily obtainable were examined.

Betaïne phosphotungstate. Betaïne is precipitated from very dilute solutions by phosphotungstic acid as a heavy white precipitate. The salt may be obtained in the form of large clear rhombohedral prisms by recrystallisation from water or dilute alcohol (Photograph 1). It is exceedingly soluble in acetone and in dilute acetone. Its composition satisfies the formula

$$(C_5H_{11}O_2N)_3, H_3PO_4, 12WO_3.$$

0·3889 g.; 32·2 cc. 0·01 *N* NH_3 = 1·2 % N
0·3893 g.; 36·95 cc. „ „ = 1·3 % N
0·4658 g.; 0·4040 g. WO_3 = 86·7 % WO_3.

Required for above formula N = 1·3 %, WO_3 = 86·2 %.

Repeated recrystallisation does not affect its composition.

Stachydrine phosphotungstate. Solutions of stachydrine salts yield a heavy, white granular precipitate with phosphotungstic acid. The salt after purification by recrystallisation forms clusters of needles and small rhombohedral prisms, often with cut edges. It is very soluble in acetone and dilute acetone. It is anhydrous and its composition agrees with the formula

$$(C_7H_{13}O_2N)_3, H_3PO_4, 12WO_3.$$

Nicotinic acid phosphotungstate is very insoluble and is therefore precipitated from great dilutions of the base. It can be obtained in a crystalline condition forming small rhombohedral prisms. It is fairly soluble in acetone.

Choline phosphotungstate. Choline is precipitated from very dilute solutions (Gulewitsch states 1 in 20,000) by phosphotungstic acid. The initial precipitate is a heavy white amorphous substance. It can be obtained in a microcrystalline form by recrystallisation from dilute alcohol. The crystals are minute rhombohedral prisms frequently possessing faceted edges.

Analysis of the purified substance:

0·3884 g.; 36·0 cc. 0·01 *N* NH_3 = 1·3 % N
0·3869 g.; 37·2 cc. „ „ = 1·4 % N.
Required for $(C_5H_{13}O_2N)_3, H_3PO_4, 12WO_3$ = 1·29 % N.

The salt remains unchanged in composition after more than one recrystallisation. It is readily soluble in acetone and dilute acetone but in other solvents is very insoluble.

Drummond and Funk [1914] record finding choline in the acetone-soluble fraction of the phosphotungstates from rice polishings.

Colamine (amino-ethyl alcohol) phosphotungstate. Barger [1914] records that colamine is not precipitated by phosphotungstic acid unless the solutions of the base are concentrated. This was found to be the case and the salt itself is very liable to re-solution in an excess of the reagent. Recrystallised from water or alcohol it forms beautiful clear rhombohedral crystals which often attain a relatively large size. The separation of choline and colamine by a method depending on the solubilities of their phosphotungstates is practicable. The latter salt is very soluble in water, alcohol or acetone.

Urea phosphotungstate. Urea is precipitated by phosphotungstic acid from its solutions, if these are not too dilute. The resulting white amorphous salt may be readily recrystallised from hot water in which it is very soluble. Large well-formed rhombohedral prisms are thus obtained which are easy to recognise (Photograph 6). The salt is readily soluble in ethyl and methyl alcohols and in acetone.

0·2002 g.; 3·4 cc. 0·1 *N* NH_3 = 2·3 % N
0·2071 g.; 4·01 cc. „ „ = 2·7 % N.
Required for $(CH_4ON_2)_3, H_3PO_4, 12WO_3$ = 2·74 % N.

Its composition is unaltered by repeated recrystallisation.

Creatinine phosphotungstate. Dilute solutions of creatinine do not always give a precipitate immediately upon the addition of phosphotungstic acid. Upon standing, however, the crystalline salt is in many cases slowly deposited. Hofmeister [1880] gives the limit of precipitability as 1 in 12,000 whilst Demjanowski [1912] obtained precipitation at a dilution of 1 in 25,000. Precipitated in this manner from dilute solutions of the base the phosphotungstate may be obtained in large well-formed crystals which are fairly characteristic. They usually consist of rhombohedra and rhombohedral prisms. When precipitated from more concentrated solutions the salt is thrown down as a heavy white precipitate consisting of microscopic needle-shaped crystals. The pure salt is anhydrous and is not very soluble in water or alcohol but is very soluble in various dilutions of acetone.

Analysis:

0·2090 g.; 5·91 cc. 0·1 N NH_3 = 4·0 % N
0·2070 g.; 5·59 cc. ,, ,, = 4·0 % N
0·1002 g.; 0·0865 g. WO_3 = 86·3 % WO_3.

Required for $(C_4H_7ON_3)_3, H_3PO_4, 12WO_3$ = N 3·92 %, WO_3 86·7 %.

Its composition is unaltered by repeated recrystallisation.

Carnosine phosphotungstate. Carnosine is precipitated from very dilute solutions as the phosphotungstate in the form of a white heavy powder. It recrystallises in very minute whetstone- and needle-shaped crystals from water or dilute alcohol. Demjanowski [1912] describes its crystalline form and records its precipitability.

Analysis:

0·2074 g.; 3·47 cc. 0·1 N NH_3 = 2·3 % N
0·2082 g.; 3·60 cc. ,, ,, = 2·4 % N.
Required for $(C_9H_{14}O_3N_4)_3, 2H_3PO_4, 24WO_3$ = 2·6 % N.

Its composition is unaltered by purification It is very soluble in acetone and dilute acetone.

Guanidine phosphotungstate is very insoluble in water and is precipitated from very dilute solutions of guanidine. When recrystallised from water or dilute alcohol it usually separates out again as a white microcrystalline powder consisting of minute spherulites of needles. Small rhombohedra are also sometimes obtained.

0·3904 g.; 11·49 cc. 0·1 N NH_3 = 4·1 % N
0·3879 g.; 11·36 cc. ,, ,, = 4·1 % N
0·3715 g.; 0·3461 g. WO_3 = 93·2 % WO_3.

Required for $(CH_5N_3)_3, H_3PO_4, 12WO_3$ = 4·16 % N and 90·5 % WO_3.

It is very soluble in acetone and mixtures of acetone and water.

Methylguanidine phosphotungstate is a heavy white powder which by recrystallisation from water or alcohol yields crystals consisting of small rhombohedral prisms with cut edges. Demjanowski [1912] describes a similar appearance for the salt. It is precipitated from very dilute solutions of methylguanidine salts.

(e) *Inorganic Phosphotungstates.*

A few inorganic salts of phosphotungstic acid are of importance from the point of view of this research, since the inorganic bases they contain are frequently present in tissue extracts or other material under investigation. The most important of these salts are the phosphotungstates of sodium, potassium and ammonium.

Ammonium, Sodium and Potassium phosphotungstates. Sodium phosphotungstate is very soluble in water and can only be obtained by concentration of its solutions almost to dryness.

Potassium phosphotungstate on the other hand is very slightly soluble in water and still less so in 5 % sulphuric acid, so that sodium and potassium may be almost quantitatively separated by this means. Ammonium phosphotungstate exhibits similar solubilities to the potassium salt, being very slightly soluble in most solvents. They are both difficult to obtain in a crystalline condition, usually occurring as very fine white precipitates which readily pass through filters.

Solubilities of the Phosphotungstates.

The solubilities in a number of solvents of all the phosphotungstates prepared during the course of this investigation have been determined, quantitatively where possible, and qualitatively where the amount of material was insufficient for the purpose, and the results are summarised in Table III.

The determinations were in all cases carried out at room temperature (18—20° C.) and are expressed in grams per 100 g. of solvent.

The figures given for the solubilities of certain of the phosphotungstates in 57 % acetone are taken from the paper by Wechsler [1911].

The original aim of the investigation, as has already been stated, was to attempt the separation of mixtures of bases by taking advantage of differences in the solubilities of their phosphotungstates. Wechsler compared the solubilities of a number of these salts in a solvent composed of four volumes of acetone to three of water and concluded that his results had a practical value. Reviewing the table of solubilities we see that wide variations in solubility are few.

The relatively great solubility of the majority of phosphotungstates in acetone is marked and this solubility becomes much greater if water is present.

The following series of solubility determinations in various dilutions of acetone were made upon a preparation of adenine phosphotungstate.

TABLE III

Phosphotungstate	Water	Ethyl alcohol	50% Ethyl alcohol	Methyl alcohol	Acetone	75 % Acetone	57 % Acetone	50 % Acetone	25 % Acetone	Remarks
Glycine	4·5	14·44	21·30	v.s.	v.s.	v.s.	—	v.s.	v.s.	—
Alanine	8·38	60·0	400·0	v.s.	v.s.	v.s.	—	v.s.	v.s.	—
Aspartic Acid	3·0	23·8	400·0	v.s.	v.s.	v.s.	—	v.s.	v.s.	—
Glutaminic Acid	f.s.	f.s.	—	v.s.	v.s.	—	—	—	—	—
Cystine	sl.s. cold, v.s. hot	sl.s. cold, v.s. hot	—	v.s.	v.s.	v.s.	—	v.s.	v.s.	—
Phenylalanine	f.s. cold	f.s.	—	v.s.	v.s.	v.s.	—	v.s.	v.s.	—
Histidine	0·4	8·8	v.s.	30·6	33·4	v.s.	160·0	v.s.	v.s.	Insoluble in ether, $CHCl_3$, CCl_4
Arginine	0·2	3·0	v.s.	28·7	25·6	v.s.	120–130	v.s.	v.s.	” ”
Lysine	0·3	3·6	f.s.	9·6	12·6	v.s.	140	v.s.	v.s.	” ”
Trimethylamine	v.sl.s.	v.sl.s.	sl.s.	sl.s.	v.s.	v.s.	—	v.s.	v.s.	” ”
Isobutylamine	v.sl.s.	sl.s.	f.s.	f.s.	v.s.	v.s.	—	v.s.	v.s.	” ”
Isoamylamine	v.sl.s.	v.sl.s.	f.s.	f.s.	v.s.	v.s.	—	v.s.	v.s.	” ”
Tripropylamine	v.sl.s.	v.sl.s.	f.s.	sl.s.	v.s.	v.s.	—	v.s.	v.s.	” ”
Triamylamine	v.sl.s.	sl.s.	f.s.	f.s.	v.s.	v.s.	—	v.s.	v.s.	” ”
Putrescine	v.sl.s.	f.s.	—	f.s.	v.s.	v.s.	—	v.s.	v.s.	” ”
Cadaverine	v.sl.s.	sl.s.	2	sl.s.	v.s.	v.s.	—	v.s.	v.s.	” ”
Phenylethylamine	sl.s.	f.s.	—	f.s.	v.s.	—	—	v.s.	—	” ”
p-Hydroxyphenylethylamine	sl.s.	f.s.	—	v.s.	v.s.	—	—	v.s.	—	” ”
β-Iminazolyl-ethylamine ...	sl.s.	f.s.	—	f.s.	v.s.	—	—	v.s.	—	” ”
Indolethylamine	v.sl.s.	f.s.	—	v.s.	v.s.	—	—	v.s.	—	” ”
Adenine	0·05	0·05	0·5	0·1	2·9	19·3	10·5	9·4	0·1	” ”
Guanine	0·05	0·08	—	1·0	2·2	—	7·21	9·0	—	” ”
Xanthine	v.sl.s.	v.sl.s.	—	sl.s.	sl.s.	—	—	f.s.	—	” ”
Hypoxanthine	v.sl.s.	v.sl.s.	—	sl.s.	sl.s.	—	—	f.s.	—	” ”
Urea	v.s.	v.s.	v.s.	v.s.	v.s.	—	—	v.s.	—	—
Creatinine	0·2	0·3	2·7	0·6	8·0	v.s.	13·34	21·5	—	—
Carnosine	v.sl.s.	sl.s.	—	f.s.	v.s.	v.s.	—	v.s.	—	—
Guanidine	0·2	0·4	19·5	29·2	9·9	v.s.	22·8	29·7	27·1	Insoluble in ether, $CHCl_3$ or CCl_4
Methylguanidine	v.sl.s.	sl.s.	—	sl.s.	f.s.	—	—	v.s.	—	” ”
Colamine	v.s.	v.s.	—	v.s.	v.s.	—	—	v.s.	—	” ”
Betaïne	0·1	0·1	2·8	1·2	30·5	v.s.	—	v.s.	v.s.	” ”
Choline	0·02	0·02	0·5	0·6	115·0	37·0	—	16·8	0·6	” ”
Stachydrine	0·05	0·03	0·8	—	23·0	v.s.	—	v.s.	3·4	” ”
Nicotinic acid	0·05	0·20	2·1	—	6·5	58·0	—	12·9	3·5	” ”
Sodium	v.s.	v.s.	—	—	—	—	—	—	—	” ”
Potassium	0·3	0·3	—	4·5	0·3	—	—	v.sl.s.	—	” ”
Ammonium	0·2	0·2	—	3·0	0·2	—	—	v.sl.s.	—	” ”

v. = very. sl. = slight. f. = fairly.

TABLE IV.

% volume acetone	Solubility
100	2·9
80	15·4
75	19·3
60	12·3
60	9·4
30	2·0
25	0·1
10	0·07
0	0·05

The greatest solubility usually occurs, as in the above case, when acetone of about 90—75 % is used.

Acetone has already been used as a means of separating phosphotungstic acid precipitates into fractions, Funk [1915] and Drummond and Funk [1914]. The purines are usually found in the acetone-insoluble fractions, an observation which is in agreement with the solubility data recorded here.

The question of the practical utility of these results is an open one and it must be left so until experience has shown their value. The separation of sodium and potassium phosphotungstates, taking advantage of the great difference in their solubilities in water, the separation of the purine bases by the insolubility of their phosphotungstates in acetone, the separation of cystine and tyrosine, a process already examined by Plimmer [1913], and the separation of choline and colamine are a few of the more obvious applications of these results.

In the earlier stages of the work it was believed that the phosphotungstates of certain substances might be used as a help to the identification of the parent base. Unfortunately this hope is now much shaken. Few of the salts examined are sufficiently characteristic in their appearance to admit of easy recognition, whilst their composition renders the value of analytical data somewhat doubtful.

Certain phosphotungstates are fairly characteristic, particularly those which possess colour. Such for example are those of adenine, guanine, uric acid, *p*-hydroxyphenylethylamine, indolethylamine, phenylalanine, phenylethylamine and cystine. Others such as creatinine, urea, and colamine may in some cases be recognised by their crystalline form. Photomicrographs of a few of the crystalline phosphotungstates are given at the end of this paper (Plate I).

The recrystallisation of mixtures of purified phosphotungstates with the object of identifying the individual components has yielded very disappointing results since the tendency to form mixed crystals is marked.

I must express my gratitude and indebtedness to Dr O. Rosenheim and Dr A. J. Ewins for their kindness in placing at my disposal pure specimens of many of the bases which have been examined during the course of this work.

SUMMARY.

1. A large number of the salts formed by amino-acids and nitrogenous bases with phosphotungstic acid have been prepared in a pure condition.

2. The composition, crystalline form and solubilities of these compounds have been examined.

3. In a few isolated cases the crystalline form or colour of the purified salt gives an indication as to the identity of the parent base, whilst separations based upon different solubilities of the salts may occasionally be made.

4. The optimum conditions for the precipitation of bases by phosphotungstic acid have been examined. The results confirm the observations of Gulewitsch that a slight excess of the precipitant operating in a medium of 5 % sulphuric acid yields the best results.

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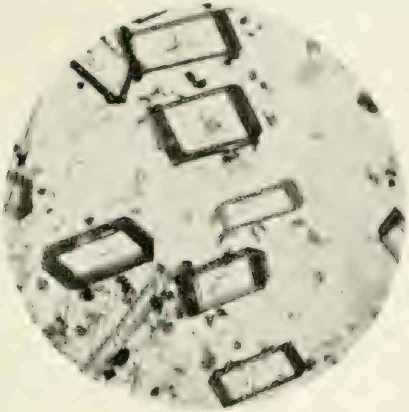
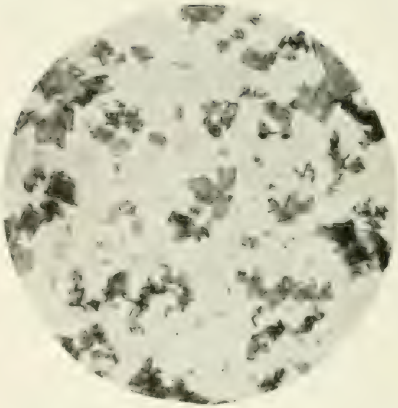
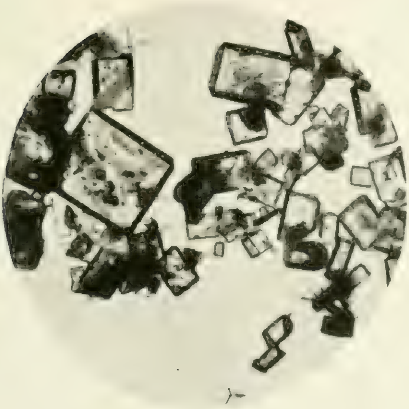


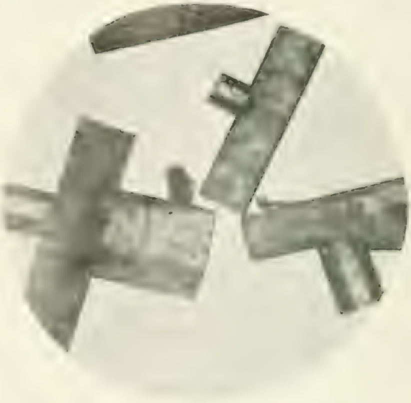
1. Betaïne Phosphotungstate.



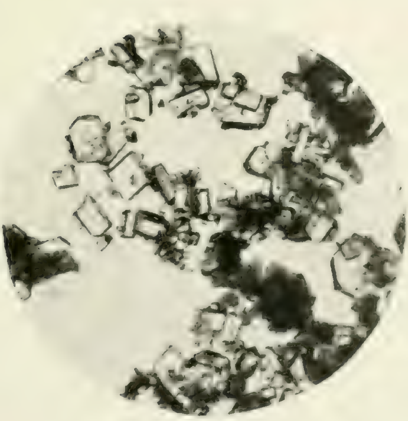
4. Cystine Phosphotungstate.



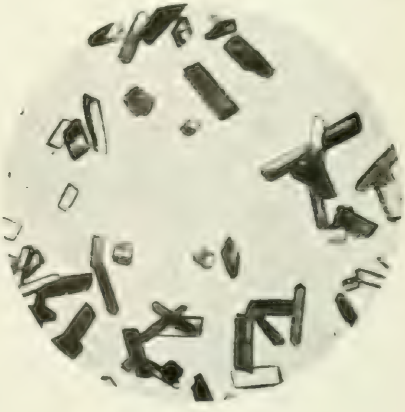
2. Adenine Phosphotungstate.



5. Arginine Phosphotungstate.



3. Histidine Phosphotungstate.



6. Urea Phosphotungstate.

Photomicrographs of some crystalline Phosphotungstates
(utilising 4 mm. objective and ocular 4. (Leitz.))

II. A STUDY OF THE WATER-SOLUBLE ACCESSORY GROWTH PROMOTING SUBSTANCE. II. ITS INFLUENCE UPON THE NUTRITION, AND NITROGEN METABOLISM OF THE RAT.

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In a previous communication it was shown that certain properties of the water-soluble accessory substance are similar to those which are associated with the so-called "anti-neuritic vitamine" [Drummond, 1917]. This fact, taken into consideration with the observation of McCollum and Kennedy [1916] that many extracts known to contain the water-soluble growth factor are active in the prevention or cure of polyneuritis in birds, led to the suggestion being advanced that the two substances are identical. If this view is correct, it cannot but be apparent from recent research how very important a rôle is played by this dietary factor in the nutrition of the animal organism. The present paper presents a series of observations bearing more or less directly upon this subject, and although they do not represent the results of a systematic research, they have been collected together in the hope that they may assist in throwing light upon certain aspects of the question.

1. *Influence of a deficiency of the water-soluble accessory factor in the diet upon the nitrogen metabolism of the rat.*

Four healthy young male rats weighing approximately 90–100 grams each were selected for this study. They were placed in pairs in metabolism cages of the type designed by Dr Alexander Paine, Director of this Institute. Modifications of this apparatus have been described [Ackroyd and Hopkins, 1916], but the original form was found to satisfy the requirements of the present investigation. By preparing the dietaries in the form of stiff pastes, the animals are prevented from scattering the food about to any appreciable extent. Fresh food and water were supplied every morning, the food-consumption being estimated by weighing the food containers before and after every 24 hour period. The 24 hours' urine was collected in a 100 cc. Erlenmeyer flask containing a few cc. of very dilute acetic acid. This, together with the cage washings, was filtered through glass-wool in order to remove fur and other extraneous matter, and made up to a known volume, usually 50 cc. The estimations were made by the usual methods; total nitrogen in faeces and urine by micro-Kjeldahl, urea by urease, ammonia by the aeration method of Folin, and creatinine and creatine by a modification of the micro-method used by Folin and Morris [1913] in their investigation of the normal protein metabolism of the rat. Two animals were fed throughout upon an artificial ration (Diet 1) which has been extensively utilised in this laboratory, and which is known to be adequate as regards all the known dietary components, since three generations of rats have been reared to maturity in the normal period upon it. The other two rats were fed for a short preliminary period upon the complete ration; then, when it had been ascertained that they were responding with a normal rate of growth, they were given Diet 2, which resembled Diet 1 in every way except for the absence of the water-soluble factor (yeast extract). The composition of the diets was as given below:

	Diet 1	Diet 2
Caseinogen ...	18 parts	18 parts
Butter-fat ...	20 ,,	20 ,,
Starch	46 ,,	52 ,,
Agar	5 ,,	5 ,,
Salt mixture ...	5 ,,	5 ,,
Yeast extract...	6 ,,	0 ,,

In order to render the diets of a pasty consistency a small quantity of distilled water was added in each case.

The tabulated results of the metabolism experiments are given in Tables I and II. These results will now be considered in detail, and other observations bearing upon each subject will be introduced where appropriate.

(*a*) *Nitrogen Metabolism.* The urinary analyses tabulated in Tables I and II do not show any striking abnormality, other than the appearance of creatinuria during the period upon the diet deficient in the water-soluble substance. This is not surprising in view of the slow decline in body-weight of the rats upon this ration caused by wasting of the skeletal muscles observed

TABLE I.

Metabolism of two normal rats fed upon a complete artificial Dietary. (Diet 1.)

Day	Body-weights g.	Rectal temperature °C.	Food consumed g.	N intake g.	Calorific intake	Total N in urine g.	% urea N	% ammonia N	% creatinine N	% creatine + creatinine N	N in faeces g.	Total N excreted g.	N balance g.
1	94 118	38·4 38·2	31·0	0·861	117	0·491	—	—	—	—	0·050	0·541	+0·320
2	—	—	32·6	0·904	123	0·603	—	—	—	—	0·041	0·644	+0·260
3	—	—	26·2	0·665	99	0·251	80·0	11·5	0·65	0·70	0·046	0·297	+0·368
4	—	—	21·1	0·538	80	0·234	—	—	—	—	0·036	0·270	+0·268
5	109 131	38·4 37·9	22·5	0·571	85	0·210	76·7	11·1	1·03	1·04	0·042	0·252	+0·319
6	—	—	23·6	0·600	89	0·233	77·7	12·0	1·03	1·03	0·033	0·266	+0·334
7	120 141	—	33·3	0·846	126	0·255	—	—	—	—	0·027	0·282	+0·564
8	—	—	29·3	0·731	111	0·320	79·2	8·5	0·81	0·86	0·043	0·363	+0·368
9	—	—	32·9	0·822	124	0·307	80·1	10·0	0·94	0·99	0·047	0·354	+0·468
10	—	—	34·6	0·865	131	0·318	76·9	8·2	0·92	0·96	0·038	0·356	+0·509
11	128 151	38·4 38·2	37·7	0·943	143	0·384	—	—	—	—	0·055	0·439	+0·504
12	—	—	35·2	0·880	132	0·425	—	—	—	—	0·049	0·474	+0·406
13	—	—	28·4	0·710	107	0·396	—	—	—	—	0·048	0·444	+0·266
14	137 160	38·4 38·3	29·8	0·745	113	0·421	80·2	8·5	0·72	0·79	0·043	0·464	+0·281
15	—	—	35·8	0·902	136	0·406	80·1	8·6	0·95	0·96	0·053	0·459	+0·443
16	—	—	29·4	0·740	111	0·380	—	—	—	—	0·041	0·421	+0·319
17	149 169	—	34·8	0·878	132	0·356	—	—	—	—	0·047	0·403	+0·475
18	—	—	36·8	0·928	139	0·478	78·2	10·0	1·12	1·16	0·030	0·508	+0·420
19	157 178	38·4 38·7	38·6	0·972	146	0·529	78·9	8·9	1·03	1·03	0·047	0·576	+0·396

Metabolism of two normal rats upon a diet deficient in water-soluble substance. (*Diet* 2.)

(*Fore and after periods on Diet* 1.)

Day	Diet	Body-weights g.		Rectal temperatures °C.		Remarks	Food consumed g.	N intake g.	Calorific intake	Total N in urine g.	% urea N	% ammonia N	% creatinine N	% creatine + creatinine N	N in faeces g.	Total N excreted g.	N balance g.
1	1	84	86	38·0	37·9	—	42·5	1·300	160	0·315	73·5	10·0	0·94	0·98	0·052	0·367	+0·933
2	1	—		—		—	28·1	0·840	107	0·310	—	—	—	—	0·033	0·343	+0·497
3	1	93	101	—		Blood examination normal ...	29·5	0·900	112	0·284	82·9	9·1	0·76	0·78	0·054	0·338	+0·562
4	1	—		—		—	31·5	0·950	119	0·252	—	—	—	—	0·044	0·296	+0·654
5	1	106	116	38·1	38·0	—	27·7	0·835	105	0·262	82·1	7·3	1·08	1·08	0·023	0·285	+0·550
6	1	—		—		Animals very fit	20·5	0·610	82	0·232	—	—	—	—	0·016	0·248	+0·362
7	2	109	119	38·3	38·3	—	12·1	0·233	46	0·285	—	—	—	—	0·022	0·307	−0·174
8	2	—		—		—	8·5	0·157	32	0·175	—	—	—	—	0·026	0·201	−0·044
9	2	107	110	38·2	38·5	—	17·6	0·356	70	0·136	80·7	6·2	0·63	0·90	0·047	0·201	+0·155
10	2	—		—		Urine dark	14·5	0·290	57	0·171	75·3	8·2	1·12	1·32	0·020	0·191	+0·100
11	2	—		—		,, ...	17·8	0·340	71	0·182	—	—	—	—	0·016	0·198	+0·142
12	2	—		—		—	14·0	0·252	56	0·171	—	—	1·19	1·36	0·040	0·210	+0·042
13	2	106	107	38·0	37·7	Urine dark	15·1	0·282	60	0·198	—	—	—	—	0·026	0·224	+0·058
14	2	—		—		,, ...	14·1	0·264	56	0·209	—	—	—	—	0·022	0·231	+0·033
15	2	—		—		Urine scanty	13·4	0·249	53	0·192	—	—	—	—	0·041	0·233	+0·016
16	2	—		—		,, ...	12·8	0·236	52	0·195	74·5	7·4	0·97	1·21	0·025	0·220	+0·016
17	2	106	107	37·6	37·3	Blood examined found normal	10·6	0·192	42	0·181	75·5	7·6	1·02	1·20	0·008	0·189	+0·003
18	2	—		—		Urine dark	12·0	0·222	47	0·194	—	—	1·03	1·13	0·025	0·219	+0·003
19	2	—		—		,, ...	8·8	0·155	35	0·178	—	—	1·20	1·54	0·045	0·213	−0·058
20	2	—		—		,, ...	10·2	0·184	41	0·178	—	—	1·24	1·69	0·017	0·195	−0·011
21	2	104	106	37·4	37·4	Animals quiet, fur ruffled ...	11·6	0·244	46	0·162	—	—	1·38	1·82	0·028	0·190	−0·054
22	2	—		—		—	9·2	0·163	37	0·156	—	—	—	—	0·014	0·170	−0·007
23	2	—		—		Animals thin and cold ...	8·8	0·290	61	0·320	72·7	7·5	0·74	1·11	0·043	0·363	−0·070
24	2	103	102	37·2	37·0	Urine scanty	6·4										
25	2	100	99	—		,, ...	7·3	0·282	58·5	0·308	—	—	—	—	0·043	0·351	−0·069
26	2	—		—		,, ...	7·3										
27	2	—		—		Animals unwell	10·0	0·499	100	0·384	—	—	—	—	0·034	0·418	+0·071
28	2	100	98	37·0	36·8	,, ,, ...	15·0										
29	2	—		—		,, ,, ...	19·0	0·389	76·2	0·405	79·0	7·3	0·66	0·85	0·058	0·463	−0·074
30	2	97	96	—		,, ,, ...											
31	2	—		—		Urine scanty	31·5	0·662	126	0·652	—	—	—	—	0·051	0·703	−0·041
32	2	97	92	—		Animals thin and cold ...											
33	2	—		—		Blood examination normal ...	18·0	0·380	72·2	0·362	79·5	8·0	0·92	1·17	0·050	0·410	−0·030
34	2	94	92	36·2	35·9	Rat 1 killed for examination											
35	1	96		36·0		Devoured new diet ravenously	13·5	0·326	48·7	0·176	—	—	—	—	0·030	0·106	+0·220
36	1	106		—		Rat much firmer and warmer	14·0	0·340	50·5	0·097	—	—	0·72	0·79	0·027	0·124	+0·216
37	1	111		37·8		—	15·5	0·376	56·0	0·173	—	—	—	—	0·024	0·197	+0·179
38	1	124		—		Rat very well	14·0	0·340	50·5	0·101	—	—	—	—	0·022	0·123	+0·217
39	1	130		38·2		—	17·5	0·425	63·2	0·186	—	—	0·82	0·84	0·019	0·205	+0·220
40	1	130		—		—	15·8	0·382	57·0	0·121	—	—	—	—	0·029	0·150	+0·232
41	1	136		38·2		Rat 2 killed for examination	14·3	0·345	51·6	0·123	—	—	—	—	0·026	0·149	+0·196

P.M. examination rat 1: The skeletal musculature was markedly wasted. There was slight enlargement of the right heart, with congestion of the kidneys and liver. Thyroid gland appeared to be smaller than normal. Other organs normal.

P.M. examination rat 2: The animal presented a normal appearance. Thyroid gland was of normal size.

during that period. Small traces of creatine are often present in rats' urine upon normal diets [Folin and Morris, 1913].

(*b*) *Body-weight.* The two control animals fed upon the complete ration (Diet 1) showed a normal rate of growth throughout the experiment. Similarly the rats studied in Table II grew at a normal rate during the preliminary and final periods, when receiving that diet. During the period when they received the diet free from the water-soluble factor (Diet 2) there was complete inhibition of growth, and a very gradual decline in body-weight. A very large number of rats have been observed whilst being fed for considerable periods upon dietaries deficient in the water-soluble substance. In the case of young rats the withdrawal of this dietary component is almost invariably followed by an immediate cessation of growth. Such inhibition is usually followed by a period during which the animal either maintains a constant body weight, or shows a very slow decline. Sooner or later there is a sudden and rapid drop in weight lasting a few days, terminated by death. If, during the periods of maintenance or decline, provided in the latter case that the condition of the rat is not extreme, the missing dietary factor is supplied in an adequate amount, the rat recovers and resumes a normal rate of growth. From a large number of observations it has been ascertained that the period of maintenance, which follows the restriction and precedes the final decline, is roughly proportional to the age of the animal when the restriction is imposed. Some average figures which illustrate this are given in Table III.

TABLE III.

Average body-weight of rat when water-soluble factor withheld from diet	Number of observations	Average duration in days of period of maintenance preceding final decline
50 g.	42	25
60 ,,	67	31
70 ,,	30	37
100 ,,	21	46
150 ,,	12	57
200 ,,	9	>60

Naturally there are wide variations to be observed in animals of all ages, but the above averages are significant of the very great importance of the water-soluble accessory substance during the earlier stages of growth. Adult animals are able to live for considerable periods without receiving a supply of this dietary factor. During this period they usually show a slow decline in body-weight, which is associated at a later stage with an enfeebled resistance to disease. The ultimate decline is, however, inevitable. From the figures given in Table III it is reasonable to assume that the need of the

TABLE IV.

*Diet deficient in water-soluble accessory substance given at *. Normal diet given at †.*

No.		Days before parturition				Days after parturition														Remarks
		8	6	4	2	0	2	4	6	8	10	12	14	16	18	20	22	24	26	
1	Wt. of Mother	126	129*	135	139	110	107	101	102	100	90	—	—	—	—	—	—	—	—	Five young born, four devoured 1st day; remaining one 3rd day. Mother died with sepsis. No attempt to rear young.
	„ Young	—	—	—	—	25	4·5	—	—	—	—	—	—	—	—	—	—	—	—	
2	Wt. of Mother	137	141*	140	137	101	107	110	117	100	107†	114	121	135	—	—	—	—	—	Five young born, three born dead. Remaining two devoured first day.
	„ Young	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
3	Wt. of Mother	150	159	160*	162	115	115	110	110	106	104	104†	114	120	124	132	132	—	—	Seven born, two killed to reduce size of litter, two died 6th day, one died 8th, 11th and 12th days.
	„ Young	—	—	—	—	25	30	31	30	19	13	—	—	—	—	—	—	—	—	
4	Wt. of Mother	175*	180	182	182	132	120	116	106	—	—	—	—	—	—	—	—	—	—	Litter born during night. All but one devoured. That one dead. Mother died with septic condition of uterus 8th day.
	„ Young	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
5	Wt. of Mother	—	—	156	160*	140	140	135	131	132	127	129	120	120	120†	117	132	137	145	Two young born.
	„ Young	—	—	—	—	20	25	32	34	36	38	38	37	34	32	34	40	54	63	
6	Wt. of Mother	—	—	186	189*	149	138	141	138	140	133	132	130	126	121†	141	144	148	154	Five young born. One died 17th day.
	„ Young	—	—	—	—	37	43	50	70	82	88	86	82	84	69	65	70	79	86	
7	Wt. of Mother	—	—	175	176*	132	128	128	128	129	123	125	120	118	111†	125	129	129	135	Nine young born, three killed to reduce size of litter.
	„ Young	—	—	—	—	36	39	43	49	67	86	94	96	92	90	94	98	101	115	
8	Wt. of Mother	—	150	163	177	147	140	140	141*	140	137	137	135	132	130	132†	139	140	142	Five young born.
	„ Young	—	—	—	—	29	34	46	52	56	62	77	72	70	64	62	62	64	70	
9	Wt. of Mother	160	168	172	186	149	148	142	140	142*	140	137	137	135	134	132	130	131	134	Six young born. Three killed to reduce size of litter.
	„ Young	—	—	—	—	19	24	35	42	50	62	67	70	74	72	70	67	60	54	

organism for the water-soluble factor during the very early stages of growth must be of an urgent nature. McCollum, Simmonds and Pitz [1916] found that the two dietary accessories, the "fat soluble A" and the "water soluble B" are only present in the milk of the nursing mother (rat) when they are supplied to her in her own dietary; it being, therefore, improbable that the animal organism can synthesise either substance. The experimental results of these authors regarding the latter substance have been confirmed by a series of experiments made in this laboratory. A number of healthy, full-grown female rats were selected from the breeding stock, all of which had reared at least one satisfactory litter previously. They were mated with reliable breeding males, and as soon as pregnancy was observed, they were removed to separate nesting cages provided with an ample supply of fine wood-shavings for bedding. At various stages, before and after parturition, individual females were placed upon the ration deficient in the water-soluble factor, and the influence of this deficiency upon the mother and young was closely noted. The results of this experiment are summarised in Table IV. From a consideration of the results contained in that table it will be seen that a restriction in the supply of the water-soluble factor in the diet of a pregnant rat may be followed by very serious consequences, both to herself and her offspring. This was particularly marked when the restriction was imposed before parturition. When imposed at, or after, delivery, the young usually lived, and in some cases even showed a subnormal rate of growth for a short period, but the fatal decline was not long in appearing. By returning the mother to a normal diet, the young could be saved, although the improvement in them was not immediately appreciable.

Further points concerning the relationship of the water-soluble factor to growth, will be considered in the later section which deals with its influence upon food-consumption.

(*c*) *Body-temperature.* The rectal temperature of the rats, whose metabolism was being studied, was taken regularly. It was found that a slow fall in body-temperature occurred in the two animals fed upon the inadequate ration. In order to confirm this observation, the body-temperatures of a large number of rats of various ages upon normal diets, and upon diets deficient in the water-soluble factor, were registered. The readings were taken at the same times during the day, as it was found that the normal rat shows considerable diurnal variation in body temperature. All the rats examined were under adult size (140 gms.), and the following average figures were obtained:

TABLE V.

Sex	Time	Average normal temperature	Average temperature after 10 days upon inadequate diet	Average temperature after 21 days upon inadequate diet
Male	10 a.m.	38·1°	37·8°	37·0°
	6 p.m.	38·5°	37·9°	37·2°
Female	10 a.m.	38·4°	38·0°	37·1°
	6 p m.	38·7°	38·3°	37·5°

(*d*) *General Health and Conditions of the Organs.* Apart from the metabolism study reported in these pages, the researches carried out in this laboratory during the last year or two have provided an opportunity for observing the state of health of a large number of rats fed upon diets deficient in the factor being considered in this paper. Whilst many of these observations were merely of a cursory nature, the general appearance of the animal alone being recorded, others were more complete, including observations of body temperature, cellular content of the blood, careful post-mortem examinations, and histological examination of the individual organs.

For some time after the water-soluble factor has been withheld from the diet the general appearance of the animals is apparently normal. They are lively, vigorous, sleek-coated and bright-eyed. As the period of maintenance at constant body-weight draws to a close, the rats gradually lose this appearance. The fur loses its gloss and becomes ruffled, whilst the rats assume a hunched attitude, and become lethargic. During the final decline the rats appear pale and emaciated, feeling cold and thin to the touch, and they usually remain huddled in corners away from the light. Funk and Macallum [1915] have recorded that rats fed upon diets deficient in the water-soluble factor (yeast) showed a condition of spastic contraction resembling avian beri-beri, or infantile tetany, 24 or 48 hours before death. Only three cases showing symptoms at all suggestive of nerve disorders have been noted in this laboratory during the careful observation of a very large number of rats upon such diets. All three cases showed a uniformity of symptoms, usually beginning with an apparent difficulty in co-ordinating the movements of the hind limbs, which appeared about 24 hours before death. The incoordination became progressively worse, until there was partial paresis of the hindquarters. As the fatal termination approached, these animals periodically passed through violent convulsive spasms lasting a minute or two. During such attacks the loss of control over the hind quarters was complete, the head was rolled from side to side, and respiration became spasmodic, being assisted by violent diaphragmatic contractions. An attempt was made to cure one rat showing these advanced symptoms, by an administration of a yeast extract, but it was unsuccessful, the animal dying an hour later.

A number of blood examinations were made upon rats during the period of maintenance. In practically every case normal values for cell counts and haemoglobin percentage were obtained. It is of interest to note that the cellular counts noted for the rats investigated in this work (black variety of *Mus norvegicus*) were uniformly lower than the average figures given for the albino rat by Taylor [1916]. His observation of the wide variations in the differential counts of normal rats was confirmed. The figures obtained are given in Table VI.

TABLE VI.

	Young adult rat normal diet; average of 12 observations		Young adult rats deficient diet; average of 14 determinations		Adult albino rat (Taylor)	
Red corpuscles	$(8–9·7) \times 10^6$		$(7·8–9·5) \times 10^6$		$(9–10·5) \times 10^6$	
White ,,	8,000–14,000		7,000–12,000		12,000–30,000	
Polymorphonuclears	30		31		50	
Large mononuclears	24	wide variation	32	wide variation	14	wide variation
Small ,,	30		29		30	
Transitional	15		7		5	
Eosinophile	1		1		1	

The cause underlying the fatal termination which invariably follows the absence of the water-soluble factor from the diet has not been ascertained. A considerable number of rats which have died from the consequences of this deficiency have been subjected to post-mortem examination. The majority presented an emaciated appearance, the wasting being limited chiefly to the skeletal musculature. Enlargement of the right heart was noted in a few cases, whilst a number of animals showed very small thyroid glands. A normal macroscopic appearance of the lungs, kidneys, pancreas, suprarenals, liver, spleen and alimentary tract was always observed. No examination of the nervous system was made. This may appear regrettable, when the possible relationship between this condition and the so-called avian polyneuritis is considered, but no opportunity for the study being made presented itself. Tissues removed from animals at post-mortem examination were subjected to histological examination. Testicles removed from adult males showed in several cases an inhibition of spermatogenesis. The results of the histological examination will furnish the subject of a separate communication.

Funk and Douglas [1914] examined the organs from a number of pigeons which had developed symptoms of the so-called polyneuritis, as a result of continued feeding upon a diet of polished rice. They recorded complete atrophy of the thymus, whilst the other glands examined all showed a diminution in size, with microscopic evidence of degenerative change in the cells with higher function. The testes were atrophied and spermatogenesis was absent. The thyroid, although atrophied, did not show abnormalities regarding the colloid content.

That inadequacies in the diet of animals may adversely influence breeding is known to all who have have had experience in feeding artificial rations.

That the inadequacy under consideration affects the male rat in this manner was shown by the following breeding trials. Six adult bucks, all known to be good breeders, were placed upon the diet deficient in the water-soluble substance (Diet 2). On the first day one animal was mated with two good breeding females who were receiving normal food, and was allowed to run with them for 48 hours. During this period the sexes were separated to receive their respective diets every morning and evening, and then allowed to run together again. Every seven days another buck was mated with two normally fed does for the same period, under exactly the same conditions. After having run with the females, each male rat was killed, the testicles being removed for histological examination. The results are given in summarised form below:

TABLE VII.

No. of male	Days upon inadequate dietary before given access to females	Result of mating
1	0	2 pregnancies, 15 young, all reared
2	7	1 pregnancy, 3 young, all reared
3	14	no pregnancy
4	21	,,
5	28	,,
6	35	,,

(e) *Food-consumption.* From the figures given in Table II, it may be observed that a marked fall in the food-consumption of the rats occurred when the diet was deficient in the water-soluble factor. This has been repeatedly observed with large numbers of other rats, although it was more apparent in some cases than in others. The significance of the calorific intake in such studies as these has been made the subject of several investigations.

Hopkins [1912] submitted this question to a very complete examination and showed conclusively that growth was inhibited in young rats, by the absence of the accessory substances, at a time when the food intake of the animals was more than sufficient quantitatively to maintain normal growth. He also found that whilst the absolute food-consumption of animals rapidly growing upon diets containing the accessory substances was greater than of those not growing upon the deficient diet, yet the consumption per unit live weight was, in comparable groups, nearly the same. He therefore concluded that any effect of the accessory substances upon the appetite must be secondary to a more direct effect upon the growth processes. The majority of investigators have observed that the absence of the water-soluble factor causes a lowered food-consumption [Funk and Macallum, 1915; Osborne and Mendel, 1917]. The latter authors have discussed the question in the light of some experiments they made, and concluded that yeast added to a diet deficient in the "water-soluble B" exerts some favourable influence upon the metabolism of the rat, causing it to eat more because its general condition is improved. This is confirmatory of the views expressed by Hopkins [1912].

A number of experiments bearing upon this subject were made during the course of the present investigation.

Two batches of twelve young rats were taken. One lot was fed upon the complete ration (Diet 1), the second batch receiving the inadequate diet (Diet 2). The food-consumption and body-weight of the animals were recorded daily, and were as follows:

Table VIII.

	Inadequate diet (3·95 cals. per g.)			Complete diet (3·34 cals. per g.)		
Day	Average wt. g.	Average food consumption g. per day	Average calorific intake per 100 g. wt. per day	Average wt. g.	Average food consumption g. per day	Average calorific intake per 100 g. wt. per day
1	89	6·6	29·4	90	13·6	50·5
5	91	6·3	27·5	110	14·3	45·0
9	90	6·7	29·4	123	15·6	42·4
13	87	6·4	29·2	135	16·7	40·2
17	86	6·0	27·6	149	17·1	38·4
21	85	5·7	26·6	156	18·0	38·6

As the calorific intake per 100 g. live weight was very low upon the inadequate diet, it was of importance to ascertain whether growth was possible upon such intake, provided that the accessory factor under consideration was present. The following ration was used:

Caseinogen ...	20 parts	Salt mixture	5 parts
Starch ...	25 ,,	Yeast extract	5 ,,
Butter-fat ...	20 ,,	Water ...	20 ,,
Agar ...	5 ,,		

The calorific value was approximately 3·7 calories per g. A series of healthy young rats were selected and weighed quantities of this food were supplied to them daily. The results are tabulated in Table IX.

TABLE IX.

	1st day	8th day	15th day	22nd day	29th day	36th day	43rd day
1. Food per day, g.*	8	8	8	8	8	20	20
Average calories per 100 g. live weight	35·2	27	24·3	22·5	21·8	52·8	45·8
Average yeast extract per 100 g. weight	0·48	0·37	0·33	0·30	0·29	1·77	1·54
Weight of rat, g.	84	109	122	132	136	140	161
2. Food per day, g.	8	8	8	8	8	24	24
Average calories per 100 g. live weight	35·2	27	24·8	22·9	22·5	63·6	55·9
Yeast extract per 100 g. live weight	0·48	0·37	0·33	0·30	0·30	0·72	0·63
Weight of rat, g.	84	110	120	130	132	139	159
3. Food per day, g.	8	8	8	8	8	20	24
Average calories per 100 g. live weight	36·6	28·8	26·5	26·1	24·4	62·0	62·5
Yeast extract per 100 g. live weight	0·48	0·38	0·36	0·36	0·33	0·78	0·71
Weight of rat, g.	81	103	112	114	122	129	142
4. Food per day, g.	8	8	4	5	10	10	10
Average calories per 100 g. live weight	37·5	31·5	16·0	19·0	36·8	33·0	28·4
Yeast extract per 100 g. live weight	0·48	0·42	0·21	0·19	0·60	0·53	0·46
Weight of rat, g.	79	95	93	97	100	112	130
5. Food per day, g.	8	8	4	5	10	10	10
Average calories per 100 g. live weight	33·9	26·5	13·5	17·4	33·0	29·8	26·8
Yeast extract per 100 g. live weight	0·46	0·36	0·18	0·23	0·54	0·48	0·43
Weight of rat, g.	88	112	110	106	112	124	138
6. Food per day, g.	8	8	4	5	10	10	10
Average calories per 100 g. live weight	32·6	24·8	13·3	17·2	33·0	30·8	28·4
Yeast extract per 100 g. live weight	0·44	0·33	0·18	0·23	0·54	0·50	0·46
Weight of rat, g.	91	120	112	108	112	120	130

* Weight of food recorded was given daily for week following.

The figures given in this table demonstrate that rats may show growth, at a corresponding period of development, upon a calorific intake lower than 29 calories per 100 g. live weight, provided that the accessory substance is supplied in adequate amount. Hopkins has stated [1912], "Only those perhaps who have had experience of feeding animals with excess of food, and have noted the amount eaten for considerable periods, will realise how well adjusted under normal circumstances is the instinctive appetite to the physiological needs."

Many opportunities for observing the truth of this statement have been met with during the course of the present research.

In the absence of the water-soluble accessory factor it is probable that the calorific intake is lowered approximately to that required for maintenance at the stage of development which has been reached. This may be illustrated by the results obtained in a series of feeding experiments designed to ascertain whether food consumption is affected by the calorific value of the diet, when the water-soluble factor "B" is absent.

The following dietaries were prepared:

	High calorific diet	Low calorific diet
Caseinogen	20 parts	20 parts
Starch	35 ,,	45 ,,
Butter-fat	30 ,,	10 ,,
Salt mixture ...	5 ,,	5 ,,
Water	10 ,,	40 ,,
Calorific value per g.	5·06	3·04

TABLE X.

	Low calorific diet (12 rats)				High calorific diet (12 rats)			
Days	Average body-weight g.	Average food consumed daily g.	Average daily calorific intake	Average calories per 100 g. live weight	Average body-weight g.	Average food consumed daily g.	Average daily calorific intake	Average calories per 100 g. live weight
0	78	6·9	21·0	32·0	76	4·2	21·2	34·2
4	79	9·0	27·3		72	5·7	28·9	
8	81	8·5	25·9		69	4·4	22·3	
12	76	8·4	25·6		69	5·2	26·4	
16	76	7·7	23·4		69	4·5	22·7	
20	74	7·6	23·1		69	5·0	25·4	
24	72	7·7	23·4		74	4·3	21·8	
28	71	8·2	24·9		69	5·1	25·9	
	Animals given (diet 1) 5 % yeast extract diet (3·37 cals. per g.)				Animals given (diet 1) 5 % yeast extract diet (3·37 cals. per g.)			
29	72	13·5	45·6	63·4	70	12·0	40·5	57·9
33	89	15·6	52·7	59·0	78	12·8	43·1	55·3
37	101	16·0	54·0	53·4	91	13·3	44·9	49·4
	Animals given modified diet 1, 5 % meat extract instead of yeast extract (3·25 cals. per g.)							
38	106	11·3	36·7	42·6	109	14·6	49·2	45·0
42	105	14·0	45·5		121	14·8	50·0	41·3
46	104	15·0	48·7		130	16·5	55·7	42·8
50	105	14·6	47·5		142	17·0	57·3	40·4
54	105	14·2	46·1		150	18·1	61·1	40·7

The food consumption of two closely similar batches of young rats fed upon these rations was noted, the results being given in Table X. It will be seen that the calorific intakes per 100 g. live weight of both groups approximate closely. The question of the influence of flavouring agents upon the palatibility of the ration and food consumption has received attention from several investigators. Both Hopkins [1912], and Osborne and Mendel [1917], have shown, by administering the accessory substances separately from the main food supply, that the action of these substances is direct upon the

TABLE XI.

	Group 1 (8 rats)			Group 2 (6 rats)			Group 3 (6 rats)			Group 4 (4 rats)		
Day	Yeast extract g. per day	Average daily food intake, g.	Average weight g.	Yeast extract g. per day	Average daily food intake, g.	Average weight g.	Yeast extract g. per day	Average daily food intake, g.	Average weight g.	Yeast extract g. per day	Average daily food intake, g.	Average weight g.
0	0	7·2	86	0·25	8·9	90	0·5	10·0	80	1·0	13·2	86
2	0	8·1	89	0·25	8·9	90	0·5	12·4	86	1·0	14·6	89
4	0	7·4	84	0·25	10·0	92	0·5	14·1	89	1·0	15·0	94
6	0	7·6	87	0·25	9·4	93	0·5	14·2	90	1·0	15·7	102
8	0	7·9	87	0·25	9·7	92	0·5	14·1	92	1·0	17·0	105
10	0	8·0	89	0·25	9·8	94	0·5	15·0	96	1·0	16·0	110
12	0	8·2	87	0·25	9·2	96	0·5	13·2	99	1·0	16·7	116
14	0	8·2	85	0·25	9·7	96	0·5	13·0	100	1·0	17·2	122
16	0	7·7	85	0·25	9·9	95	0·5	12·7	104	1·0	15·0	129
18	0	7·4	84	0·25	10·0	94	0·5	13·2	110	1·0	17·9	134
20	0	7·1	86	0·25	9·7	95	0·5	14·5	112	1·0	19·2	137
22	0	6·7	84	0·25	11·1	97	0·25	12·0	110	1·0	18·0	142
24	0·5	10·2	86	0·75	15·0	105	0·25	12·0	110	1·0	20·0	149
26	0·5	11·1	88	0·75	16·7	112	0·25	13·0	109	1·0	21·2	150
28	0·5	14·4	90	0·75	17·0	119	0·25	12·0	109	1·0	19·1	157
30	0·5	13·0	93	0·75	18·7	122	0·25	12·2	108	0	17·2	156
32	0·5	12·9	96	0·75	18·5	130	0·25	12·2	109	0	15·4	150
34	0·5	14·2	98	1·0	19·8	138	0·25	11·7	107	0	15·0	150
36	0·5	15·0	110	1·0	19·4	147	0·25	10·7	105	0	15·6	149
38	0·5	15·1	114	1·0	19·0	150	0·25	9·8	105	0	14·3	147
40	0·5	14·6	116	1·0	20·0	150	0·25	9·9	104	0	14·0	146

growth processes. Commercial meat extract has been found to be deficient in the water-soluble accessory factor [Drummond, 1917]. The influence of this substance, acting as far as is known purely as a flavouring agent, upon food-consumption may be seen from Table X. An increase in calorific intake is apparent without any growth resulting.

Osborne and Mendel [1917] found that the amount of growth is proportional to the amount of yeast fed, the other components of the diet being adequate. Some experiments of this nature were made in this laboratory, mainly with the object of determining the minimum quantity of extract containing the accessory factor which would give growth. Four series of healthy young rats were selected, and placed upon the diet deficient in the water-soluble factor. A standard yeast extract was then prepared, measured quantities of which were administered *per os* to each rat daily by means of a small syringe before the animals received their morning meal. The results are tabulated in Table XI.

From Table XI the following average figures have been extracted (Table XII).

Table XII.

Approximate amount of yeast extract per 100 g. live weight given daily	Result	Animals yielding result
0	Maintenance or slow decline	Groups 1 and 4
0·25	Maintenance	Groups 2 and 3
0·50	Slow growth	Groups 1 and 3
0·60	Medium growth	Groups 1 and 3
0·70	Good growth	Group 2
1·0—1·5	Rapid growth	Group 4

These figures show reasonable agreement with some of the other results recorded in these pages. Thus, from Table IX, it will be seen that several rats receiving 8 g. of diet daily, containing 0·4 g. yeast extract, showed cessation of growth upon reaching an average weight of 135 g. that is when ingesting approximately 0·30 g. yeast extract per 100 g. body weight.

Although, as Hopkins has pointed out [1912], it is unwise to expend time upon determinations of this nature until a more or less pure substance has been isolated, it may yet be of interest to note certain facts regarding the composition of the yeast extract. The 0·3 g. contained 0·21 g. of solids, of which approximately 70 % was represented by products known to be inactive as regards growth-accelerating influence, such as inorganic salts. The remaining 0·064 g. contained the accessory factor under consideration, as well as considerable amounts of adenine and leucine.

2. *Concerning the presence of the Water-soluble Accessory Substance in actively growing Animal Tissues.*

The water-soluble accessory substance has not been found in association with extracts of animal tissues, such for example as commercial meat extract. Likewise fresh beef tissue has been found during the present research to be deficient in the factor. The question arose as to whether it might be present in tissues in the process of rapid growth. To test this, concentrated protein-free aqueous extracts were prepared from three such tissues, (*a*) rat-embryos, 30 days old; (*b*) rat sarcoma tissue; (*c*) Rous chicken sarcoma tissue. In no case did the administration of these extracts, or the feeding of the fresh tissues themselves correct the inadequacy of the diet deficient in the water-soluble B (Table XIII).

TABLE XIII.

Nature and percentage of substance added to inadequate diet	Average weight of 6 rats every seven days					Average period in days of maintenance before final decline
	0	7	14	21	28	
Inadequate ration alone	64	63	67	63	54	33
4 % beef tissue	56	59	62	59	49	31
5 % commercial meat extract	86	87	84	80	80	46
6 % aqueous extract rat embryo	58	60	63	56	52	30
10 % ,, ,, rat sarcoma ...	60	66	58	52	—	27
10 % ,, ,, fowl sarcoma ...	56	50	51	51	49	30
1 % desiccated pituitary gland	47	45	49	50	52	—
5 % desiccated pituitary gland	52	56	50	48	47	—
1 % desiccated anterior lobe pituitary ...	64	66	70	67	60	46
3 % desiccated anterior lobe pituitary ...	74	73	72	71	67	40
2 % desiccated thyroid gland	60	61	56	52	—	24
5 % desiccated thymus gland	77	78	71	70	70	40
2 % desiccated testicular tissue	46	45	52	47	41	30
2 % desiccated ovarian tissue	70	64	60	—	—	29

3. *Relationship of the Water-soluble Substance to the Glands of Internal Secretion.*

More than one author has made the suggestion that the accessory substances exert their influence upon growth by being indispensable for the functionation of one or more of the glands of internal secretion [Hopkins, 1912; Funk, 1914].

During the present investigation several such glands were added in the desiccated form to the diet deficient in the water-soluble accessory and administered to young rats, in order to determine whether any one of them would supply the missing factor. As may be seen from Table XIII, no such result was obtained in any case.

Summary.

1. The nitrogen metabolism of rats fed upon an artificial diet deficient in the water-soluble accessory substance has been studied. The only apparent deviation from the normal noted was the appearance of creatinuria. It was accompanied by a slow wasting of the skeletal muscles.

2. The food consumption of rats fed upon the deficient diet is low, being in all probability reduced to that sufficient to supply the calorific requirements of maintenance.

3. An increased food consumption may be brought about by the addition of flavouring agents such as meat extract to the diet, but unless this agent contains the water-soluble substance no growth results.

4. The addition to the inadequate diet of extracts containing the water-soluble substance causes a greatly increased food intake, immediately followed by growth.

5. The amount of growth is, within certain limits, proportional to the amount of accessory substance added, provided that the diet is adequate in other respects.

6. There is evidence that the length of time that an animal (rat) is able to maintain itself upon a diet deficient in the water-soluble substance without suffering a serious loss of body-weight is directly proportional to the age at which the restriction is imposed.

7. Actively growing animal tissues (embryos, tumours) do not contain appreciable amounts of the "water-soluble B."

8. Desiccated pituitary gland (whole gland and anterior lobe), thyroid, thymus, testicle and ovarian tissues are also deficient in the "water-soluble B."

9. The cause of the fatal decline which inevitably follows a deficiency of the water-soluble substance has not been determined. Symptoms of nerve disorder were noted before death in three cases.

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III. SUR LA DIGESTION DES PROTÉINES DE LA VIANDE CUITE CHEZ DES CHIENS À CAROTIDES LIÉES.

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DANS un but d'économie, j'ai été amené à utiliser, au cours de recherches sur la digestion de la viande cuite de bœuf, des chiens de 9 à 10 kilogrammes, auxquels, pour d'autres motifs, on avait prélevé à différentes reprises du sang hors de l'une, puis hors de l'autre carotide, en l'espace de trois à quatre semaines. Par suite de ces saignées, on a été obligé de lier plusieurs fois au cou chacune des deux carotides. On a effectué avec la plus stricte aseptie les divers prélèvements de sang et les ligatures des carotides. Chaque saignée a comporté 120 à 150 cc. On a pratiqué la dernière 8 à 10 jours avant l'ingestion de viande. Les plaies du cou nécessitées par les saignées ont parfaitement guéri par première intention sans le moindre symptôme morbide local ou général. Les animaux présentaient un excellent appétit, n'avaient en aucune façon perdu en poids, étaient très gais, et paraissaient en parfaite santé.

Entre la dernière saignée et l'ingestion de viande, on a soumis les chiens au traitement antihelminthique habituel. On les a maintenus 24 à 36 heures à jeûn, tout en leur permettant de boire de l'eau à volonté. Ils ont alors reçu 25 grammes de viande cuite de bœuf par kilogramme de poids. On les a sacrifiés respectivement 3, 7, 8, 9 et 10 heures après ce repas. On a procédé au prélèvement des contenus du corps de l'estomac, de la portion prépylorique de cet organe et de la première partie de l'intestin grêle de la façon décrite dans la communication sur la digestion des protéines de la viande de cuite bœuf, récemment parue dans ce journal [Zunz, 1915]. On a suivi pour la séparation de l'azote coagulable et de l'azote non coagulable, pour la détermination de la répartition de l'azote non coagulable entre les principaux groupes de dérivés des protéines, pour le dosage de l'azote ammoniacal et de l'azote aminé aliphatique dans chacun de ces contenus, exactement la même technique que dans les recherches effectuées chez des chiens normaux.

Or, à mon grand étonnement, j'ai bientôt constaté que l'évacuation de l'estomac s'effectuait plus lentement chez des chiens à carotides liées que chez des animaux non soumis au préalable à cette intervention opératoire. Il était, par conséquent, interdit de tenir compte lors de l'étude de la digestion normale de la viande cuite de bœuf, des résultats obtenus chez les cinq chiens à carotides liées. J'avais l'intention d'étudier de façon détaillée la digestion de la viande chez des chiens à carotides liées et de rechercher si les saignées répétées exercent ou non une influence sur les processus digestifs, en dehors de celle de la ligature des carotides. Mais, par suite de circonstances indépendantes de ma volonté, il me sera sans doute impossible de m'occuper d'ici à longtemps de cette question. Ceci m'engage à communiquer dès à présent les quelques données expérimentales recueillies sur la digestion des protéines de la viande cuite chez des chiens à carotides liées.

Les Tableaux I à IV résument les constatations effectuées chez des chiens à carotides liées. Ils indiquent 1° la quantité d'azote ingérée; 2° les quantités d'azote existant encore à l'état de protéines intactes dans le corps de l'estomac, dans la région prépylorique de cet organe et dans tout l'estomac; 3° les quantités d'azote trouvées sous forme d'acidalbumine, de protéoses et d'autres dérivés des substances albuminoïdes dans les deux portions de l'estomac, dans l'ensemble de cet organe et dans toute la première partie de l'intestin grêle; 4° les quantités d'azote ammoniacal et d'azote aminé aliphatique renfermées dans les trois portions du tube digestif examinées; 5° les quantités d'azote disparues de l'estomac.

On a calculé les données des Tableaux I à III en % de l'azote ingéré (Tableau V), et en % de l'azote contenu dans la région de l'estomac examinée (Tableau VI), celles des Tableaux I à IV en % de l'azote incoagulable renfermé dans la portion du tube digestif envisagée (Tableau VII).

Même au bout de dix heures, le corps de l'estomac et la portion prépylorique de cet organe renferment des quantités appréciables de bol alimentaire de telle sorte qu'on peut examiner séparément le contenu de chacune des deux portions de cet organe, alors que chez les chiens normaux ceci n'est plus possible dès la septième heure consécutive à l'ingestion de viande cuite de bœuf.

Le nombre des expériences effectuées chez des chiens à carotides liées est beaucoup trop faible pour permettre d'établir des moyennes bien fondées. On doit plutôt se borner à comparer les résultats obtenus au bout des mêmes

TABLEAU I.

Chien en expérience	Poids de l'animal, en grammes	Durée de la digestion, en heures	Quantité de viande ingérée, en grammes	Azote ingéré, en grammes	Azote trouvé dans le corps de l'estomac											
					en grammes										en milligrammes	
					à l'état coagulable	à l'état incoagulable								total	à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
						sous forme d'acid-albumine	sous forme de protéoses			sous forme d'autres produits			total			
							hétéro-albumose et proto-albumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total				
M	10200	3	255	8·8206	4·6628	0·0082	0·6459	0·2948	0·9407	0·1589	0·1511	0·3100	1·2589	5·9217	5·68	7·82
N	9000	7	225	8·1016	1·7338	0·3306	0·2994	0·1934	0·4928	0·2312	0·1804	0·4116	1·2350	2·9688	21·60	37·62
O	9700	8	242·5	9·4114	1·0600	0·0188	0·2690	0·3331	0·6021	0·4001	0·2976	0·6977	1·3186	2·3786	49·84	75·43
P	10400	9	260	10·6288	1·9740	0·0797	0·1787	0·1437	0·3224	0·2273	0·0485	0·2758	0·6779	2·6519	23·31	21·86
Q	8750	10	219·25	8·3052	0·7633	0·0192	0·1023	0·3448	0·4471	0·0437	0·0719	0·1156	0·5819	1·3452	22·27	11·88

TABLEAU II.

Chien en expérience	Durée de la digestion, en heures	Azote trouvé dans la région prépylorique de l'estomac											
		en grammes										en milligrammes	
		à l'état coagulable	à l'état incoagulable								total	à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
			sous forme d'acid-albumine	sous forme de protéoses			sous forme d'autres produites			total			
				hétéro-albumose et proto-albumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total				
M	3	0·0470	0·0004	0·0218	0·0281	0·0499	0·0150	0·0147	0·0297	0·0800	0·1270	2·98	3·24
N	7	0·1640	0·0234	0·0412	0·0360	0·0772	0·1016	0·0322	0·1338	0·2344	0·3984	4·14	20·78
O	8	0·1044	0·0012	0·0073	0·0142	0·0215	0·0364	0·0491	0·0855	0·1082	0·2126	2·89	6·08
P	9	0·1523	0·0198	0·0452	0·0296	0·0748	0·0520	0·0152	0·0672	0·1618	0·3141	4·82	7·22
Q	10	0·0605	0·0008	0·0046	0·0145	0·0191	0·0129	0·0257	0·0386	0·0585	0·1190	3·12	4·84

TABLEAU III.

Chien en expérience	Durée de la digestion, en heures	Azote trouvé dans l'estomac total												
		en grammes										en milligrammes		
		à l'état coagulable	à l'état incoagulable								total	à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique	Azote disparu de l'estomac, en grammes
			sous forme d'acid-albumine	sous forme de protéoses			sous forme d'autres produits			total				
				proto-albumose et hétéro-albumose	autres protéoses	total	precipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total					
M	3	4·7098	0·0086	0·6677	0·3229	0·9906	0·1739	0·1658	0·3397	1·3389	6·0487	8·66	11·06	2·7719
N	7	1·8978	0·3540	0·3406	0·2294	0·5700	0·3328	0·2126	0·5454	1·4694	3·3672	25·74	58·40	4·7344
O	8	1·1644	0·0200	0·2763	0·3473	0·6236	0·4365	0·3467	0·7832	1·4268	2·5912	52·73	81·51	6·8202
P	9	2·1263	0·0995	0·2239	0·1733	0·3972	0·2793	0·0637	0·3430	0·8397	2·9660	28·13	29·08	7·6628
Q	10	0·8238	0·0200	0·1069	0·3593	0·4662	0·0566	0·0976	0·1542	0·6404	1·4642	25·39	16·72	6·8410

TABLEAU IV.

Chien en expérience	Durée de la digestion, en heures	Azote trouvé dans la première portion de l'intestin grêle à l'état incoagulable									
		en grammes								en milligrammes	
		sous forme d'acid albumine	sous forme de protéoses			sous forme d'autres produits			total	à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
			hétéro-albumose et proto-albumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total			
M	3	0·0000	0·0037	0·0260	0·0297	0·0308	0·0476	0·0784	0·1081	6·58	7·50
N	7	0·0020	0·0012	0·0026	0·0038	0·0279	0·0461	0·0740	0·0798	15·75	18·71
O	8	traces	0·0019	0·0034	0·0053	0·0478	0·0947	0·1425	0·1478	13·38	12·79
P	9	,,	0·0033	0·0086	0·0119	0·0513	0·0561	0·1074	0·1193	5·95	11·88
Q	10	0·0000	0·0046	0·0197	0·0243	0·0618	0·0840	0·1458	0·1701	15·47	16·90

Tableau V.

Chien en expérience	Durée de la digestion, en heures	Portion de l'estomac examinée	Pour-cent de l'azote ingéré retrouvés dans l'estomac												disparus de l'estomac
			à l'état coagulable	à l'état incoagulable								en tout	à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique	
				sous forme d'acid-albumine	sous forme de protéoses			sous forme d'autres produits			total				
					proto-albumose et hétéro-albumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total					
M	3	corps	52·86	0·09	7·33	3·34	10·67	1·80	1·71	3·51	14·27	67·13	0·07	0·09	—
		région prépylorique	0·53	0·01	0·24	0·32	0·56	0·17	0·17	0·34	0·91	1·44	0·03	0·04	—
		estomac total ...	53·39	0·10	7·57	3·66	11·23	1·97	1·88	3·85	15·18	68·57	0·10	0·13	31·43
N	7	corps	21·40	4·08	3·69	2·39	6·08	2·85	2·23	5·08	15·24	36·64	0·27	0·46	—
		région prépylorique	2·02	0·29	0·52	0·44	0·96	1·26	0·39	1·65	2·90	4·92	0·05	0·26	—
		estomac total ...	23·42	4·37	4·21	2·83	7·04	4·11	2·62	6·73	18·14	41·56	0·32	0·72	58·44
O	8	corps	11·26	0·20	2·86	3·54	6·40	4·25	3·16	7·41	14·01	25·27	0·53	0·80	—
		région prépylorique	1·11	0·01	0·08	0·15	0·23	0·39	0·52	1·01	1·15	2·26	0·03	0·07	—
		estomac total ...	12·37	0·21	2·94	3·69	6·63	4·64	3·68	8·42	15·16	27·53	0·56	0·87	72·47
P	9	corps	18·57	0·75	1·68	1·35	3·03	2·14	0·46	2·60	6·38	24·95	0·22	0·21	—
		région prépylorique	1·44	0·18	0·43	0·28	0·71	0·49	0·14	0·63	1·52	2·96	0·04	0·06	—
		estomac total ...	20·01	0·93	2·11	1·63	3·74	2·63	0·60	3·23	7·90	27·91	0·26	0·27	72·09
Q	10	corps	9·19	0·23	1·23	4·16	5·39	0·53	0·86	1·39	7·01	16·20	0·27	0·14	—
		région prépylorique	0·73	0·01	0·06	0·17	0·23	0·15	0·31	0·46	0·70	1·43	0·04	0·07	—
		estomac total ...	9·92	0·24	1·29	4·33	5·62	0·68	1·17	1·85	7·71	17·63	0·31	0·21	82·37

TABLEAU VI.

			Pour-cent de l'azote contenus dans la région de l'estomac examinée										
			en grammes									en milligrammes	
Chien en expérience	Durée de la digestion, en heures	Portion de l'estomac examinée	à l'état coagulable	à l'état incoagulable								à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
				sous forme d'acid-albumine	sous forme de protéoses			sous forme d'autres produits			total		
					hétéro-albumose et proto-albumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total			
M	3	corps	78·74	0·14	10·91	4·98	15·89	2·68	2·55	5·23	21·26	0·09	0·13
		région prépylorique	37·01	0·32	17·16	22·13	39·29	11·81	11·57	23·38	62·98	2·35	2·55
		estomac total ...	77·86	0·14	11·04	5·36	16·40	2·86	2·74	5·60	22·14	0·14	0·18
N	7	corps	58·40	11·13	10·09	6·51	16·60	7·79	6·08	13·87	41·60	0·73	1·27
		région prépylorique	41·16	5·88	10·34	9·04	19·38	25·50	8·08	33·58	58·84	1·04	5·22
		estomac total ...	56·36	10·51	10·12	6·81	16·93	9·88	6·32	16·20	43·64	0·76	1·73
O	8	corps	44·56	0·79	11·31	14·00	25·31	16·83	12·51	29·34	55·44	2·09	3·17
		région prépylorique	49·11	0·56	3·43	6·63	10·11	17·12	23·09	40·21	50·89	1·36	2·86
		estomac total ...	44·94	0·77	10·66	13·41	24·07	16·84	13·38	30·22	55·06	2·03	3·11
P	9	corps	74·44	3·01	6·73	5·42	12·15	8·57	1·83	10·40	25·56	0·88	0·82
		région prépylorique	48·49	6·30	14·39	9·42	16·56	23·81	4·84	21·40	51·51	1·53	2·30
		estomac total ...	71·68	3·36	7·55	5·84	13·39	9·42	2·15	11·57	28·31	0·95	0·98
Q	10	corps	56·74	1·43	7·60	25·64	32·24	3·25	5·34	8·59	42·26	1·66	0·88
		région prépylorique	50·84	0·67	3·87	12·18	16·05	10·84	21·60	32·44	49·16	2·62	4·07
		estomac total ...	56·26	1·37	7·30	24·54	31·84	3·86	6·67	10·53	43·74	1·73	1·14

TABLEAU VII.

Chien en expérience	Durée de la digestion, en heures	Portion du tube digestif examinée	Pour-cent de l'azote incoagulable contenus dans la région du tube digestif examinée								
			sous forme d'acid-albumine	sous forme de protéoses			sous forme d'autres produits			à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
				hétéroalbumose et protoalbumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total		
M	3	corps de l'estomac	0·65	51·32	23·40	74·72	12·63	12·00	24·63	0·46	0·62
		région prépylorique	0·50	27·25	35·13	62·38	18·75	18·37	37·12	3·83	4·50
		estomac total ...	0·64	49·88	24·11	73·99	12·99	12·38	25·37	0·65	0·83
		intestin grêle ...	0·00	3·42	24·05	27·47	28·49	44·04	72·52	6·09	6·94
N	7	corps de l'estomac	26·77	24·24	15·66	39·90	18·72	14·61	33·33	1·75	3.05
		région prépylorique	9·98	17·58	15·36	32·94	43·34	13·74	57·08	1·77	8·87
		estomac total ...	24·09	23·18	15·61	38·79	22·65	14·47	37·12	1·75	3·97
		intestin grêle ...	2·51	1·50	3·26	4·76	34·96	57·77	92·73	19·74	23·45
O	8	corps de l'estomac	1·42	20·40	25·27	45·67	30·34	22·57	52·91	3·78	5·73
		région prépylorique	1·11	6·75	13·12	19·87	33·64	45·38	79·02	2·67	5·62
		estomac total ...	1·40	19·37	24·34	43·71	30·59	24·30	54·89	3·70	5·71
		intestin grêle ...	0·00	1·29	2·30	3·59	32·34	64·07	96·41	9·05	8·65
P	9	corps de l'estomac	11·76	26·36	21·20	47·56	33·53	7·15	40·68	3·44	3·22
		région prépylorique	12·24	27·94	18·29	46·23	32·14	9·39	41·53	2·92	4·46
		estomac total ...	11·85	26·66	20·64	47·30	33·26	7·59	40·85	3·35	3·46
		intestin grêle ...	0·00	2·77	7·21	9·98	43·00	47·02	90·02	5·99	9·96
Q	10	corps de l'estomac	3·30	17·58	59·25	76·83	7·51	12·36	19·87	3·83	2·04
		région prépylorique	1·37	7·86	24·79	32·65	22·05	43·93	65·98	5·33	8·27
		estomac total ...	3·12	16·70	56·10	72·80	8·84	15·24	24·08	3·97	2·61
		intestin grêle ...	0·00	2·70	11·58	14·28	36·33	49·38	85·72	8·51	9·94

laps de temps chez les chiens normaux et chez les chiens à carotides liées. Le Tableau VIII permet d'établir cette comparaison pour ce qui concerne l'azote ingéré, existant soit à l'état coagulable, soit à l'état incoagulable dans l'estomac et pour la rapidité de l'évacuation de cet organe. Quant à l'examen séparé des deux portions de l'estomac, la comparaison n'est naturellement possible que pour les chiens sacrifiés 3 et 7 heures après le repas.

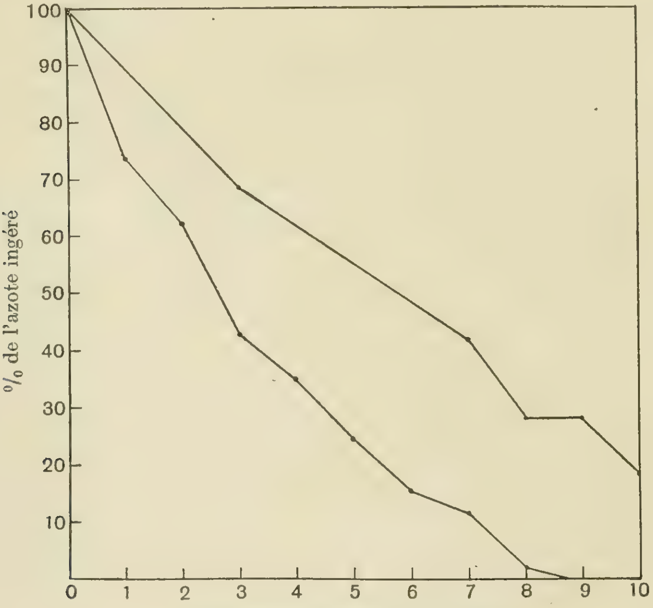
Au bout de ces laps de temps, on trouve, chez les chiens à carotides liées, une quantité beaucoup plus considérable d'azote coagulable et d'azote incoagulable dans le contenu du corps de l'estomac que chez les chiens normaux. Il n'y a pas de différences bien nettes sous ce rapport entre les deux catégories d'animaux pour le contenu de la portion prépylorique de l'estomac. Si l'on examine l'ensemble de cet organe, on observe des quantités bien plus notables d'azote à l'état de protéines intactes ou de leurs dérivés chez les chiens à carotides liées que chez les autres. Cette différence entre les deux catégories d'animaux est bien plus marquée pour l'azote coagulable 7 à 10 heures après l'ingestion de viande cuite de bœuf que 3 heures après ce repas.

L'azote coagulable l'emporte sur l'azote incoagulable dans le contenu du corps de l'estomac et dans celui de cet organe tout entier, sauf chez le chien O, sacrifié 8 heures après l'ingestion de viande cuite de bœuf. Dans le contenu de la portion prépylorique de l'estomac, l'azote incoagulable l'emporte sur l'azote coagulable chez les chiens M et N, sacrifiés 3 et 7 heures après le repas; les quantités d'azote coagulable et d'azote incoagulable sont par contre à peu près égales chez les trois autres animaux à carotides liées.

Chez les chiens normaux, l'azote coagulable existe toujours en quantité moindre que l'azote incoagulable à partir de la sixième heure consécutive à l'ingestion de viande cuite de bœuf dans le contenu du corps de l'estomac et dans celui de cet organe considéré dans sa totalité. Quant au contenu de la portion prépylorique de l'estomac, il renferme davantage d'azote coagulable que d'azote incoagulable, sauf chez le chien F, sacrifié 6 heures après le repas.

Le Tableau VIII et le tracé ci-contre montrent nettement que l'évacuation de l'estomac s'effectue plus lentement chez les chiens à carotides liées que chez les autres. Au bout de dix heures, on est arrivé chez les premiers environ au même stade qu'au bout de six chez les seconds.

Si l'on se base sur les chiffres d'azote trouvés à l'état coagulable et incoagulable dans l'estomac et sur les proportions d'azote ingéré disparues de cet organe, il semble que les processus digestifs atteignent leur plus grande intensité et que l'évacuation de l'estomac s'effectue le plus rapidement non pas pendant les premières heures consécutives au repas comme chez les chiens normaux, mais plutôt à une époque se rapprochant dans une certaine mesure de la septième à la huitième heure consécutive à l'ingestion de viande cuite de bœuf. Bien entendu, il ne faut pas perdre de vue qu'on ne dispose jusqu'à présent que de quelques données expérimentales à propos des chiens à carotides liées. Il ne s'agit pour le moment que de présomptions, que des recherches ultérieures effectuées chez de tels animaux, sacrifiés 1, 2, 4, 5, 6, 11 heures et davantage après l'ingestion de viande cuite de bœuf, devront confirmer ou non. On ne doit pas non plus oublier qu'il n'y a pas moyen de tenir compte de l'azote déversé avec les sucs digestifs dans l'estomac et de l'azote résorbé par cet organe, de telle sorte qu'on ne dispose pas, en réalité, de chiffres tout à fait précis, relatifs soit aux quantités d'azote ingéré existant à un moment donné à l'état coagulable et incoagulable dans ce viscère, soit même à la proportion d'azote ingéré ayant disparue alors de l'estomac.



Heures après ingestion de viande.

La proportion d'azote incoagulable à l'état d'acidalbumine a été relativement considérable dans le contenu du corps de l'estomac de deux des cinq chiens à carotides liées (chiens N et P); il est vrai qu'un chien normal a présenté un chiffre presque aussi élevé (chien F, sacrifié au bout de 6 heures). Le contenu de la région prépylorique de l'estomac a aussi montré une forte teneur en acidalbumine deux fois sur cinq (chiens N et P). La proportion d'azote incoagulable à l'état d'acidalbumine a même été, chez le chien P, plus grande dans la portion prépylorique de l'estomac que dans le corps de cet organe. Chez les autres chiens à carotides liées, la teneur en acidalbumine a au contraire diminué du corps de l'estomac à la région prépylorique de cet organe. L'acidalbumine a entièrement fait défaut deux fois sur cinq dans le contenu de la première portion de l'intestin grêle; deux fois on n'en a constaté que des traces. Chez le chien N, chez lequel le contenu du corps de l'estomac et celui de la portion prépylorique de cet organe ont présenté des teneurs relativement considérables en acidalbumine, on a observé dans le contenu de la première partie de l'intestin grêle une teneur en acidalbumine encore appréciable, bien que notablement inférieure à celle de la portion prépylorique de l'estomac.

Tableau VIII.

Laps de temps écoulé depuis l'ingestion de viande, en heures	Portion de l'estomac examinée	Etat des chiens	Pour-cent de l'azote ingéré		
			existants dans l'estomac à l'état		disparus de l'estomac
			coagulable	non-coagulable	
3	corps ...	normaux ...	45·61	2·81	—
		à carotides liées	52·86	14·27	—
	portion prépylorique	normaux ...	1·48	1·38	—
		à carotides liées	0·53	0·91	—
	estomac total	normaux ...	47·09	4·19	48·77
		à carotides liées	53·39	15·18	31·43
7	corps ...	normaux ...	1·70	2·84	—
		à carotides liées	21·40	15·24	—
	portion prépylorique	normaux ...	1·37	0·96	—
		à carotides liées	2·02	2·90	—
	estomac total	normaux ...	3·07	3·80	93·13
		à carotides liées	23·42	18·14	58·44
8	estomac total	normaux ...	1·08	4·98	93·94
		à carotides liées	12·37	15·16	72·47
9	estomac total	normaux ...	0·20	3·65	96·15
		à carotides liées	20·01	7·90	72·09
10	estomac total	normaux ...	0·45	2·23	97·32
		à carotides liées	9·92	7·71	82·37

Les protéoses représentent, dans le contenu du corps de l'estomac, les 3/4 environ de l'azote incoagulable chez les chiens M et Q, moins de la moitié chez les trois autres animaux à carotides liées, alors qu'on trouve toujours les 3/5 et dans la moitié des cas plus des 4/5 de l'azote incoagulable à cet état chez les chiens normaux. Dans le contenu de la portion prépylorique de l'estomac, moins de la moitié de l'azote incoagulable existe sous forme de protéoses 4 fois sur 5, c'est à dire dans 80 % des cas, chez les animaux à carotides liées, mais seulement 2 fois sur 7, c'est-à-dire dans 28, 57 % des cas, chez les chiens normaux. Les albumoses représentent moins de 10 % de l'azote incoagulable dans le contenu de la première portion de l'intestin grêle 3 fois sur 5, c'est-à-dire dans 60 % des cas, chez les chiens à carotides liées et par contre 3 fois sur 10, soit dans 30 % des cas, chez les autres. La proportion d'azote incoagulable à l'état de protéoses diminue du contenu du corps de l'estomac à celui de la région prépylorique de cet organe; cette diminution est parfois très accusée (chiens O et Q), parfois seulement très faible (chien P). Elle est toujours fort intense du contenu de la portion prépylorique de l'estomac à celui de la première partie de l'intestin grêle.

Les protéines précipitées par demi-saturation au moyen du sulfate de zinc l'emportent sur les autres: dans le contenu du corps de l'estomac, 3 fois sur 5, soit dans 20 % des cas, chez les chiens à carotides liées, au lieu de 3 fois sur 7 chez les chiens normaux; dans le contenu de la portion prépylorique de l'estomac, 2 fois sur 5, soit dans 40 % des cas, chez les chiens à carotides liées, au lieu de 1 fois sur 7, soit dans 14·28 % des cas, chez les chiens normaux; dans le contenu de tout l'estomac 3 fois sur 5, soit dans 60 % des cas, chez les chiens à carotides liées, au lieu de 3 fois sur 10, soit dans 30 % des cas, chez les chiens normaux.

La proportion d'azote incoagulable à l'état d'hétéro-albumose et de protoalbumose diminue du contenu du corps de l'estomac à celui de la portion prépylorique de cet organe et de celui-ci à celui de la première partie de l'intestin grêle, sauf chez le chien P, sacrifié au bout de 9 heures, chez lequel on rencontre une proportion un peu plus élevée de ces produits dans la région prépylorique de l'estomac que dans le corps de cet organe.

La proportion d'azote incoagulable à l'état de protéoses non précipitées par demi-saturation au moyen du sulfate de zinc diminue aussi au fur et à mesure qu'on progresse dans le tube digestif. Toutefois, chez le chien M, il y a davantage d'azote sous cette forme dans la région prépylorique de l'estomac que dans le corps de cet organe. De plus, chez ce même animal, le contenu de la première partie de l'intestin grêle offre une proportion notablement moindre de ces composés que le contenu de la région prépylorique de l'estomac, mais dépassant néanmoins celle du contenu du corps de l'estomac.

On rencontre dans le contenu du corps de l'estomac des chiens à carotides liées 20 à 50 % de l'azote incoagulable à l'état de peptones et de polypeptides, alors que ces substances n'atteignent jamais 20 % de cet azote chez les animaux normaux nourris de la même façon. Dans le contenu de la région prépylorique de l'estomac, on trouve, tant chez les animaux à carotides liées que chez les autres, 35 à 80 % de l'azote incoagulable sous cette forme. Néanmoins chez le chien normal F, sacrifié 6 heures après l'ingestion de viande cuite de bœuf, les peptones et les polypeptides ne représentent pas le quart de l'azote incoagulable. Dans le contenu total de l'estomac, la proportion d'azote incoagulable à l'état de composés autres que l'acidalbumine et les protéoses varie entre 25 et 55 % chez les chiens à carotides liées. Chez les animaux normaux, l'estomac considéré dans son ensemble ne contient pas dans la majorité des cas (6 fois sur 10) le 1/5 de l'azote incoagulable sous forme de peptones et de polypeptides; ces produits correspondent 2 fois (chiens E et J) environ au quart et deux fois (chiens C et I) à peu près au tiers de l'azote incoagulable.

Quant à la première partie de l'intestin grêle, les composés autre que l'acidalbumine et les protéoses, c'est-à-dire les peptones, les peptides et les acides aminés dépassent toujours 70 % et souvent même 90 % de l'azote incoagulable tant chez les chiens à carotides liées que chez les autres. Chez le chien G, non opéré et sacrifié 7 heures après l'ingestion de viande cuite de bœuf, on n'observe toutefois que 68 % de l'azote incoagulable à cet état dans le contenu intestinal.

La proportion d'azote incoagulable à l'état de dérivés des protéines autres que l'acidalbumine et les protéoses s'accroît, chez les chiens à carotides liées comme chez les animaux normaux, du contenu du corps de l'estomac à celui de la portion prépylorique de cet organe et de celui-ci au contenu de la première partie de l'intestin grêle. L'accroissement est, il est vrai, insignifiant du corps de l'estomac à la région prépylorique chez le chien P, à carotides liées, sacrifié 9 heures après l'ingestion de viande cuite de bœuf.

Parmi les dérivés des protéines autres que l'acidalbumine et les protéoses, ceux précipités par l'acide phosphotungstique l'emportent sur ceux non précipités par ce réactif: dans le contenu du corps de l'estomac, 4 fois sur 5, soit dans 80 % des cas, chez les chiens à carotides liées, au lieu de 3 fois sur 7, soit dans 42·86 % des cas, chez les chiens normaux; dans le contenu de la portion prépylorique de l'estomac, 3 fois sur 5, soit dans 60 % des cas, chez les chiens à carotides liées, au lieu de 2 fois sur 7, soit dans 28·57 % des cas, chez les chiens normaux; dans le contenu de tout l'estomac, 4 fois sur 5, soit dans 80 % des cas, chez les chiens à carotides liées, au lieu de 4 fois sur 10, soit dans 40 % des cas, chez les chiens normaux; jamais dans le contenu de la première partie de l'intestin grêle chez les chiens à carotides liées, au lieu de 3 fois sur 10, soit dans 30 % des cas, chez les chiens normaux. La réaction du biuret a toujours été positive dans le contenu des trois régions du tube digestif examinées. Les peptones vraies de Kühne n'ont donc jamais fait défaut dans l'estomac et dans la première partie de l'intestin grêle des 5 chiens à carotides liées, sacrifiés respectivement 3, 7, 8, 9 et 10 heures après l'ingestion de viande cuite de bœuf.

Chez les chiens M et Q, sacrifiés 3 et 10 heures après l'ingestion de viande cuite de bœuf, la proportion d'azote incoagulable à l'état de peptones et d'autres composés précipités par l'acide phosphotungstique mais pas par le sulfate de zinc (polypeptides pour l'estomac, polypeptides et acides aminés pour l'intestin grêle) s'accroît du contenu du corps de l'estomac à celui de la portion prépylorique de cet organe et de celui-ci au contenu de la première partie de l'intestin grêle. Chez les chiens N et O, sacrifiés respectivement 7 et 8 heures après le repas, la portion prépylorique de l'estomac renferme une plus forte proportion d'azote incoagulable sous forme de ces produits que l'intestin. Chez le chien O, les trois régions du tube digestif examinées offrent, du reste, à peu près la même proportion d'azote incoagulable à l'état de composés précipités par l'acide phosphotungstique mais pas par le sulfate de zinc. Chez le chien P enfin, sacrifié au bout de 9 heures, la région prépylorique de l'estomac renferme un peu moins d'azote incoagulable sous cette forme que le corps de cet organe et beaucoup moins que la première partie de l'intestin grêle.

Quant à la teneur en composés non précipités par l'acide phosphotungstique, elle s'accroît au fur et à mesure qu'on progresse dans le tube digestif. Il faut toutefois remarquer qu'elle est un peu plus faible, chez le chien N, dans la portion prépylorique de l'estomac que dans le corps de cet organe.

Le Tableau IX facilite la comparaison de la répartition moyenne de l'azote incoagulable entre l'acidalbumine, les protéoses et les autres dérivés des protéines dans les diverses régions du tube digestif chez les chiens à carotides liées et chez les animaux normaux après l'ingestion de viande cuite de bœuf.

Il résulte nettement des chiffres relatés dans le Tableau IX qu'on constate en moyenne davantage d'azote incoagulable sous forme d'acidalbumine d'une part, de dérivés non précipités par le sulfate de zinc d'autre part, moins par contre sous forme de protéoses, tant précipitées que non précipitées par demi-saturation au moyen du sulfate de zinc, dans les diverses portions examinées du canal gastrointestinal chez les chiens à carotides liées que chez les autres.

Les composés précipités par l'acide phosphotungstique mais pas par le sulfate de zinc représentent dans chacune des deux portions de l'estomac en

TABLEAU IX.

Portion du tube digestif examinée	Etat des chiens	Pour-cent de l'azote incoagulable en moyenne sous forme						
		d'acid-albumine	de protéoses			d'autres produits		
			hétéroalbumose et protoalbumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total
corps de l'estomac...	normaux ...	4·33	42·14	41·32	83·46	6·81	5·40	12·21
	à carotides liées	8·78	27·98	28·96	56·94	20·54	13·74	34·28
portion prépylorique de l'estomac	normaux ...	3·73	19·72	30·87	50·59	18·27	27·41	45·68
	à carotides liées	5·04	17·47	21·34	38·81	29·99	26·15	56·15
estomac total ...	normaux ...	4·10	34·17	41·01	75·18	10·29	10·43	20·72
	à carotides liées	8·22	27·16	28·16	55·32	21·67	14·79	36·46
première portion de l'intestin grêle	normaux ...	0·03	6·76	11·25	18·01	36·94	45·02	81·96
	à carotides liées	0·50	2·34	9·68	12·02	35·02	52·46	87·48

TABLEAU X.

Portion du tube digestif examinée	Etat des chiens	Azote ammoniacal, en pour-cent				Azote aminé aliphatique, en pour-cent			
		de l'azote incoagulable		de l'azote total		de l'azote incoagulable		de l'azote total	
		chiffres extrêmes	moyenne	chiffres extrêmes	moyenne	chiffres extrêmes	moyenne	chiffres extrêmes	moyenne
corps de l'estomac...	normaux ...	0·03 à 3·25	1·40	0·01 à 2·03	0·58	1·25 à 7·09	3·93	0·15 à 2·51	1·27
	à carotides liées	0·46 à 3·83	2·65	0·09 à 2·09	0·89	0·62 à 5·73	2·93	0·13 à 3·17	1·26
portion prépylorique de l'estomac	normaux ...	0·15 à 5·89	3·25	0·06 à 3·24	1·55	3·13 à 13·65	7·38	1·50 à 3·82	2·57
	à carotides liées	1·77 à 5·33	3·30	1·04 à 2·62	1·78	4·46 à 8·87	6·34	2·30 à 5·22	3·40
estomac total ...	normaux ...	0·05 à 4·04	2·21	0·01 à 3·79	1·38	1·29 à 7·45	3·53	0·22 à 2·38	1·46
	à carotides liées	0·65 à 3·97	2·68	0·14 à 2·03	0·92	0·83 à 5·71	3·32	0·18 à 3·11	1·43
première portion de l'intestin grêle	normaux ...	3·21 à 8·68	6·17	—	—	5·02 à 28·82	16·60	—	—
	à carotides liées	5·99 à 9·74	9·88	—	—	6·94 à 23·45	11·77	—	—

moyenne une proportion plus considérable de l'azote incoagulable chez les chiens à carotides liées que chez les autres, tandis qu'au contraire dans l'intestin grêle, il y a une proportion légèrement moindre de ces composés chez les animaux opérés que chez les non opérés. Quant aux produits non précipités par l'acide phosphotungstique, ils comprennent une proportion plus notable de l'azote incoagulable chez les chiens à carotides liées que chez les autres dans le contenu du corps de l'estomac, dans le contenu total de cet organe et dans le contenu de la première partie de l'intestin grêle, un proportion un peu moindre au contraire chez les premiers que chez les seconds dans la région prépylorique de l'estomac.

Arrivons-en à l'azote ammoniacal et à l'azote aminé aliphatique. La teneur en chacune de ces deux espèces d'azote s'accroît, chez les chiens à carotides liées comme chez les autres, du contenu du corps de l'estomac à celui de la portion prépylorique de cet organe et de ce dernier à celui de la première partie de l'intestin grêle. Chez le chien N, sacrifié 7 heures après l'ingestion de viande cuite de bœuf, la teneur en azote ammoniacal est néanmoins à peu près la même dans les deux portions de l'estomac. Chez les chiens O et P, sacrifiés respectivement 8 et 9 heures après ce repas, la teneur en azote ammoniacal est même inférieure dans la région prépylorique de l'estomac à celle constatée dans le corps de cet organe. Chez le chien P, la portion prépylorique de l'estomac a une teneur en azote aminé aliphatique très légèrement inférieure à celle constatée dans le corps de cet organe.

L'azote aminé l'emporte sur l'azote ammoniacal sauf dans le contenu intestinal chez le chien O, dans le contenu du corps de l'estomac chez le chien P, dans le contenu total de l'estomac et dans celui du corps de cet organe chez le chien Q.

Le Tableau X permet de comparer les teneurs en azote ammoniacal et en azote aminé aliphatique des diverses régions étudiées du tube digestif après l'ingestion de viande cuite de bœuf chez les chiens à carotides liées d'une part et chez les animaux non opérés d'autre part.

Si l'on calcule la teneur moyenne en azote ammoniacal par rapport à l'azote incoagulable, on constate qu'elle est plus élevée dans les deux portions de l'estomac et dans la première partie de l'intestin grêle chez les chiens à carotides liées que chez les autres. Tel est encore le cas par rapport à l'azote total pour le contenu du corps de l'estomac et pour celui de la portion prépylorique de cet organe considéré isolément. Par contre, pour le contenu total de l'estomac, on trouve moins d'azote ammoniacal en moyenne chez les animaux opérés que chez les autres. Cette discordance tient aux résultats des chiens normaux H à J, sacrifiés 8 à 10 heures après l'ingestion de viande cuite de bœuf. Chez ces 3 animaux, on a dû se borner à examiner le contenu total de l'estomac et celui-ci possédait une teneur relativement élevée en azote ammoniacal. Or, comme par suite du ralentissement de l'évacuation de l'estomac chez les chiens à carotides liées, on n'a pu examiner des stades aussi avancés des processus digestifs, une comparaison parfaite est impossible entre les deux catégories d'animaux.

La teneur en azote aminé aliphatique, calculée par rapport à l'azote incoagulable, est moindre dans les trois parties du tube digestif examinées chez les chiens à carotides liées que chez les animaux normaux. Si l'on prend comme base de calcul l'azote total, la teneur en azote aminé est sensiblement la même dans le corps de l'estomac et dans l'ensemble de cet organe chez les deux catégories d'animaux: elle est par contre plus considérable chez les chiens à carotides liées que chez les autres dans la portion prépylorique de l'estomac.

Il ne faut pas pour le moment attacher trop d'importance aux différences signalées entre les chiens à carotides liées et les autres pour ce qui concerne les teneurs en azote ammoniacal et en azote aminé aliphatique des diverses régions du tube digestif, car on n'a pas jusqu'à présent examiné un nombre suffisant d'animaux opérés.

En définitive, ce qui caractérise surtout la digestion de la viande cuite de bœuf chez les chiens à carotides liées, c'est le ralentissement de l'évacuation de l'estomac. Ce ralentissement pourrait fort bien être dû à une moindre sécrétion de suc gastrique dans l'estomac. Cannon [1907] a, en effet, prouvé que l'ouverture et la fermeture du pylore sont placées sous le contrôle du degré d'acidité du contenu de l'antre prépylorique d'une part, du contenu duodénal d'autre part.

Les modifications essentielles constatées dans la répartition de l'azote incoagulable après ingestion de viande cuite de bœuf chez les chiens à carotides liées par rapport aux animaux normaux consistent, ainsi que nous l'avons déjà vu, en une moindre proportion de protéoses et en une proportion plus élevée d'acidalbumine et des autres dérivés des protéines. Ces différences entre chiens normaux et chiens à carotides liées sont beaucoup plus marquées dans le contenu du corps de l'estomac que dans celui de la portion prépylorique de cet organe et surtout que dans celui de la première portion de l'intestin grêle. Ces différences dépendent nécessairement du ralentissement de l'évacuation de l'estomac et des motifs de ce ralentissement: moindre sécrétion de suc gastrique ou sécrétion d'un suc moins acide que normalement.

modifications dans les mouvements de l'estomac. Ce sont là des choses à examiner ultérieurement.

A l'heure actuelle nous ignorons complètement les causes réelles des modifications éventuelles de l'activité chimique et mécanique de l'estomac. Il se peut qu'à la suite de la ligature des deux carotides, la circulation ou l'innervation de l'estomac ou toutes deux subissent de façon directe ou indirecte, peut-être seulement au bout d'un certain laps de temps, des modifications et que celles-ci entraînent à leur tour des variations dans le chimisme de l'estomac ou dans les manifestations motrices de cet organe au cours des processus digestifs. Mais il s'agit là de considérations purement hypothétiques. Il appartient à des recherches futures d'apporter la lumière sur ces points.

D'autres problèmes encore s'imposent à notre attention. Si l'on ne procédait qu'à une seule reprise à la ligature de chacune des deux carotides, observerait-on les mêmes effets qu'après les ligatures répétées de ces deux vaisseaux. Ce n'est pas absolument certain, car il semble bien d'après l'examen des carotides liées à plusieurs reprises qu'elles ont subi des modifications dans leur structure (épaississement, induration, etc.) dans un rayon plus ou moins considérable aux environs de ces ligatures, modifications qui ne semblent pas apparaître de suite et que la simple inspection visuelle ne décèle pas.

On peut aussi se demander si les saignées successives ont exercé une influence. On sait, en effet, qu'une saignée abondante entraîne diverses modifications de la composition du sang: hyperglycémie [Claude Bernard, 1887; v. Mering, 1877; Schenk, 1894; Rose, 1903], hypercholesterinémie [Mauriac, 1912], hyperaminoacidémie [György, 1914; Gorchkoff, Grigorieff et Koutoursky, 1914; György et E. Zunz, 1915].

Mais ces modifications signalées soit peu après la saignée, soit quelques heures après celle-ci, dépendent surtout des processus de régénération destinés à ramener le sang à sa concentration et à sa composition habituelle, dont l'organisme tend toujours à maintenir la constance. Elles ont sans doute cessé leurs effets au bout de 8 à 10 jours, moment où l'on a étudié la digestion de la viande cuite de bœuf chez les chiens à carotides liées. Il est donc très probable que le ralentissement de l'évacuation de l'estomac et les autres particularités observées chez les cinq chiens à carotides liées sont uniquement la conséquence de cette intervention.

Résumé.

Après ingestion de viande cuite de bœuf, l'évacuation de l'estomac s'effectue plus lentement chez les chiens à carotides liées que chez les chiens normaux. Ce phénomène et les facteurs dont il dépend, entraînent certaines modifications par rapport à la normale dans la composition du contenu du corps de l'estomac, à un moindre degré dans celle du contenu de la portion prépylorique de cet organe, et à un moindre degré encore dans celle du contenu de la première partie de l'intestin grêle. Ces modifications consistent essentiellement en une moindre proportion d'azote incoagulable à l'état de protéoses et en une plus forte proportion tant sous forme d'acidalbumine que sous celle d'autres dérivés des protéines (peptones et polypeptides pour l'estomac; peptones polypeptides et acides aminés pour l'intestin grêle).

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IV. THE VISCOSITY OF BLOOD.

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I. Introductory.

Several attempts have been made to correlate the viscosity of blood with the number of corpuscles present [Denning and Watson, 1906; Josué and Maturier, 1916]. All observers agree that the viscosity rises with increases in the number of corpuscles present and that the effect becomes progressively greater with each such increase above a certain critical value. It has also been noticed that blood from different animals shows different viscosities even when the plasma viscosity and the number of corpuscles are the same in each. These observations suggested that the factor contributed by the corpuscles depended on their total volume rather than on their number [McClendon, 1916, p. 180]. Ferrai [quoted by Denning and Watson, 1906] also, who showed that the addition of carbon dioxide to blood caused a rise in viscosity, attributed the rise to a simultaneous increase in the volume of the corpuscles. Thus a qualitative relationship has been established between the total volume of the corpuscles, the viscosity of plasma and the viscosity of whole blood; this paper is an endeavour to make clear the quantitative relationships.

II. Methods.

Home-made capillary viscosimeters were used of the pattern illustrated in Fig. 1 *a*. They are blown from tubing of 0·8 mm. diameter for the large size, using 2 cc., and 0·2 mm. diameter for the small form, holding 1 cc. In the large model the bulb *C* holds rather less than 1 cc. The other dimensions are in proportion. The funnel *D* is made from a half-inch test-tube drawn out and fused on, and holds about five cubic centimetres. It is necessary to have it of that size to prevent overflow of bubbles, which may form when the blood is blown out of *C* as described later. Two cc. of blood mixed with a little hirudin is placed in *D* and allowed to run into *C*. The instrument is then placed upright in a thermostat at 40° for ten minutes. The bulb *C* is then emptied into *D* by blowing slowly down the rubber tube *E* until the whole of the blood is in *D*. The corpuscles are mixed with the plasma by blowing a gentle stream of air through or by stirring with a coiled wire[1]. The bubbles which form are destroyed by touching them with a hot needle and the time of flow from *B* to *A* noted with a stopwatch, the blood in *D* being continually stirred with a coiled wire.

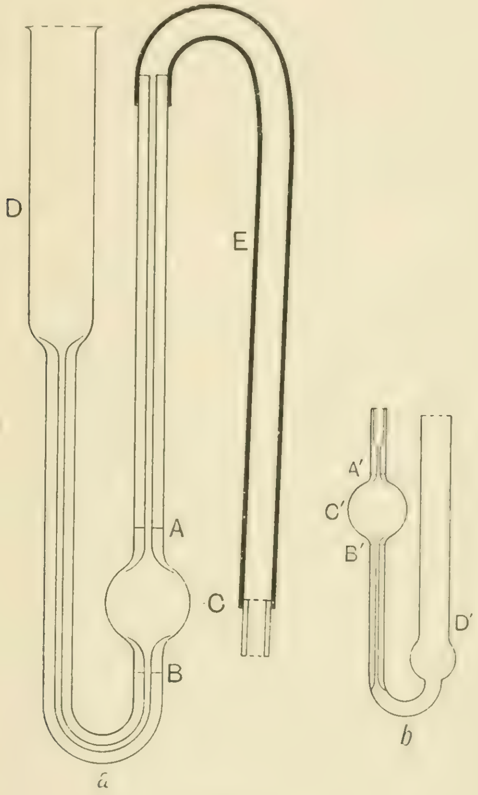

Fig. 1.

III. Causes of Error.

(*a*) *Conditions setting up turbulent flow.* The transition between the expanded portions and the capillary itself must be a gradual one and especially must not be constricted. One instrument constructed led to much waste of time because of this latter failing.

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[1] Blowing air through is of course inadmissible when the effect of small changes in the CO_2 content of the blood is in question but all the experiments to be described have been conducted with the blood fully oxygenated, and the changes in viscosity dealt with are very much greater (by as much as 1000 %) than the small changes produced by alterations in the viscosity of the blood.

Not much emphasis is laid on this point in the usual textbook descriptions of viscosity measurements. The magnitude of the error is shown by the following figures (Table I) showing the relation of the time of flow of 7 % gum acacia solution in 2 % sodium chloride (used as having a viscosity similar to that of normal blood) to the time of flow of water at different temperatures and the ratio which gives the "apparent" viscosity.

TABLE I.

A *is a viscosimeter with a constriction,* B *one without.*

	A			B		
	Time of flow			Time of flow		
Temp.	Water	Gum	Ratio	Water	Gum	Ratio
40°	12·5	43·5	3·4	10	43	4·3
16·5°	19	82	4·3	—	—	—
11·5°	—	—	—	18·0	7·9	4·45

On blowing out the constriction the results were concordant. The error was caused by the formation of eddies at the constriction. Turbulent flow was set up and the amount of energy absorbed by the eddies increases with the velocity of flow and is greater the less viscous the fluid [Osborne Reynolds, 1883]. Consequently the time of flow is increased more for the less viscous fluid and especially at the higher temperatures where the velocities of flow are higher. The "apparent" viscosity of very much more viscous fluids such as the gum solution used in the above experiment is therefore less than the real. Viscosimeters of large bore (greater than 0·5 mm.) should always be calibrated by measuring not only the time of flow of water but also that of gum solutions of known viscosities (as measured in a very fine bore viscosimeter) including the whole range of viscosities to be measured. The use of very fine bore viscosimeters for the measurement of the viscosity of blood introduces chances of large errors due to settling of the corpuscles.

(*b*) *The sedimentation of the corpuscles.* Settling of corpuscles during the course of the transpiration has not attracted the attention it deserves. The rate varies enormously in different species and considerably in different individuals of the same species. The differences depend on no single physical factor. One of the most striking features is the slow settling which sometimes takes place in saline. The size of the error can be seen in the following table.

TABLE II.

Time of flow of sheep's corpuscles in saline; haematocrit volume 30 %.

Sedimentation prevented	Sedimentation not prevented
Time of flow	Time of flow
33·4, 33·2, 33·8	35·8, 35·8 (error ca. 10 %)

This suspension settled rather rapidly. The error with a larger proportion of corpuscles (longer times of flow) is a much greater one, for the greater the number of corpuscles present the greater the rate of increase of the viscosity with further increase in concentration (see p. 68). Without stirring I have found it impossible to get concordant results with the higher concentrations of corpuscles used. Nor does the fact that the later portions of the suspension to pass through the capillary tube contain a smaller number of corpuscles than the earlier compensate for this effect: for the transference of a volume of corpuscles from a dilute suspension to a stronger one produces a greater alteration of the viscosity of the strong suspension than of the dilute one (see p. 68). This may be crudely illustrated by the fact that it takes longer to empty a room full of people if everybody jostles around the doorway than if each waits his turn.

The form of viscosimeter usually adopted, with the measuring bulb on the pressure side of the viscosimeter ($A'B'$, Fig. 1 *b*) prevents any stirring during the course of the experiments. This led I think to the differences in "apparent" viscosity which Denning found between large tubes and small. I have been unable to obtain these differences working with tubes of 0·8 mm. and 0·2 mm. diameter. The narrower tubes in their experiments produced a long time of outflow which gave greater opportunities for settling and the higher the concentration reached in any experiment the greater the deviation from the real time of flow, the percentage error increasing during the experiment.

(*c*) *Formation of beads of fluid.* On making measurements with the instrument (Fig. 1 *a*) care must be taken that beads of fluid do not collect in the capillary above A (Fig. 1 *a*). These form from the fluid, which wets the sides of the capillary, if C is emptied too rapidly into D. A similar bead may form at B and persisting as a bubble on the top of the inflowing blood, may pass into the capillary above A as a bead. These beads of fluid require very large amounts of energy to drive them along capillary tubes, especially if they contain air bubbles (cf. air embolism in the coronary arteries): and when they reach the open end of the tube a bubble forms and breaks several times. Both these processes increase the time of flow very considerably. In the type of viscosimeter, Fig. 1 *b*, these beads forming there at A' are even more troublesome.

(*d*) *Temperature variations* produce alterations in the viscosity of blood and the alterations are not parallel with that of water: all comparisons were therefore made at the same temperature, viz. 40°.

(*e*) *Use of anticoagulants.* In these experiments hirudin was used. A few fine flakes were placed in the bottom of a short test-tube into which 5 cc. of blood were run. The hirudin was without appreciable influence on the viscosity, as was shown by collecting fowl's blood directly from an artery into a clean glass vessel, measuring its viscosity, adding hirudin and measuring the viscosity again. Defibrination is inadmissible because of its effect on the plasma. Oxalate, citrate and fluoride in the solid state are also inadmissible because of the alteration in the volume of the corpuscles by osmotic changes. The following table gives the alterations produced by the addition of 0·08 % NaCl (corresponding to 0·1 % potassium oxalate and 0·35 % sodium citrate) in the corpuscular volume [Hamburger, 1905] and the consequent alteration in viscosity calculated on the assumption that the plasma viscosity remained constant (see formula established later, p. 68).

TABLE III.

Corpuscular volume		Viscosity as a multiple of water		
before adding salt	after adding salt 0·08 %	before adding salt	after adding salt	% error
33·3 %	36·7 %	5·0	4·61	9·8 %
48·6 %	53·1 %	6·6	5·92	10·1 %
58·3 %	64·1 %	9·06	7·85	12·4 %

On this point see also Burton-Opitz [1911]. In employment of potassium oxalate other complications arise. At certain concentrations potassium oxalate produces large increases in the viscosity of the plasma, large enough sometimes to mask the effect mentioned above. Sodium citrate does not appear to produce this effect. The addition of isotonic solutions of sodium citrate to blood produces no osmotic changes, but causes difficulty by diminishing the viscosity of the plasma to an unknown extent. If the volume of the corpuscles is known, the true viscosity can be calculated by one of the formulae given later, but the method is obviously very cumbrous.

(*f*) *Haemolysis* by bacteria and saponin produces very large rises in viscosity. Centrifuged corpuscles on being allowed to stand in the air, become converted into a tarry mass which will not pour at all. Saponin also produces haemolysis with rise of viscosity (Table IV).

TABLE IV.

Time of flow	
64·4 secs.	before adding saponin, 0·020 g.
90·0 ,,	immediately after
276·0 ,,	five minutes later

The rise is proportional to the amount of saponin added as is shown in Table V.

TABLE V.

Time after adding saponin	Time of flow	
0	33·5 secs.	0·005 g. dry saponin added
1½ mins.	70·0 ,,	
4½ ,,	85·0 ,,	
20 ,,	83·0 ,,	
22 ,,		0·005 g. dry saponin added
35 ,,	115·0 ,,	

The viscosity is said by Burton-Opitz to be diminished by laking produced by freezing and thawing; but the condition which is most likely to be met with in the body is more akin to that of haemolysis by saponin.

IV. MEASUREMENT OF THE CORPUSCULAR VOLUME.

The measurement of the volume of the corpuscles was carried out by centrifuging to a constant volume in a graduated tube, such as a haematocrit tube, with an ordinary electrical centrifuge working at 2500–3500 revolutions per minute. The graduated tube was closed by putting a piece of paper on the end and dipping into melted paraffin. A much firmer joint was thus made, than the leather pad and screw clip generally supplied. The time for complete sedimentation varies but is usually less than twenty-five minutes. The figures given in the following pages were obtained by centrifuging for fifty minutes. These measurements were necessarily made at room temperature, but by immersing the haematocrit tube in water at 40° it was found that the proportion between the volume of the corpuscles and of the plasma remained the same.

V. THE CONTRIBUTION OF THE PLASMA TO THE VISCOSITY OF THE BLOOD.

The viscosity of the blood is directly proportional to that of the plasma, for the same volume of corpuscles, over a very wide range of plasma viscosity (Table VI and Fig. 2).

TABLE VI.

Variation of the viscosity of suspensions of corpuscles with variation in the viscosity of an artificial plasma. Plasma: 7 % *gum acacia in* 0·9 % *saline. Corpuscles: sheep, washed in saline.*

Amount of suspension of corpuscles	0·9 % saline	7 % gum in saline	Viscosity		$\frac{b}{p}$
			plasma (p)	blood (b)	
2 cc.	1·5 cc.	0·5 cc.	1·40	3·22	2·30
2 ,,	1·0 ,,	1·0 ,,	1·77	4·06	2·29
2 ,,	·5 ,,	1·5 ,,	2·34	5·36	2·29
2 ,,	0 ,,	2·0 ,,	3·21	7·73	2·41

The table gives the result of increasing the viscosity of the plasma in a suspension of corpuscles in saline by the addition of gum acacia. Sheep's corpuscles were washed in 0·9 % saline and centrifuged until a fairly concentrated suspension was obtained. This was mixed with varying proportions of 0·9 % saline and 7 % gum acacia in 0·9 % saline as indicated in the table. The viscosity of the various mixtures was determined, as well as that of the "plasma" after the corpuscles had settled. The results are shown and the ratio between "plasma" viscosity and "blood" viscosity given. The constancy of the ratio is obvious while the values of the viscosity plotted against each other lie along a straight line intersecting the abscissa at 0. The value of the ratio $\frac{b}{p}$ (see Table VI) depends on the volume of the corpuscles present, the influence of which is next considered.

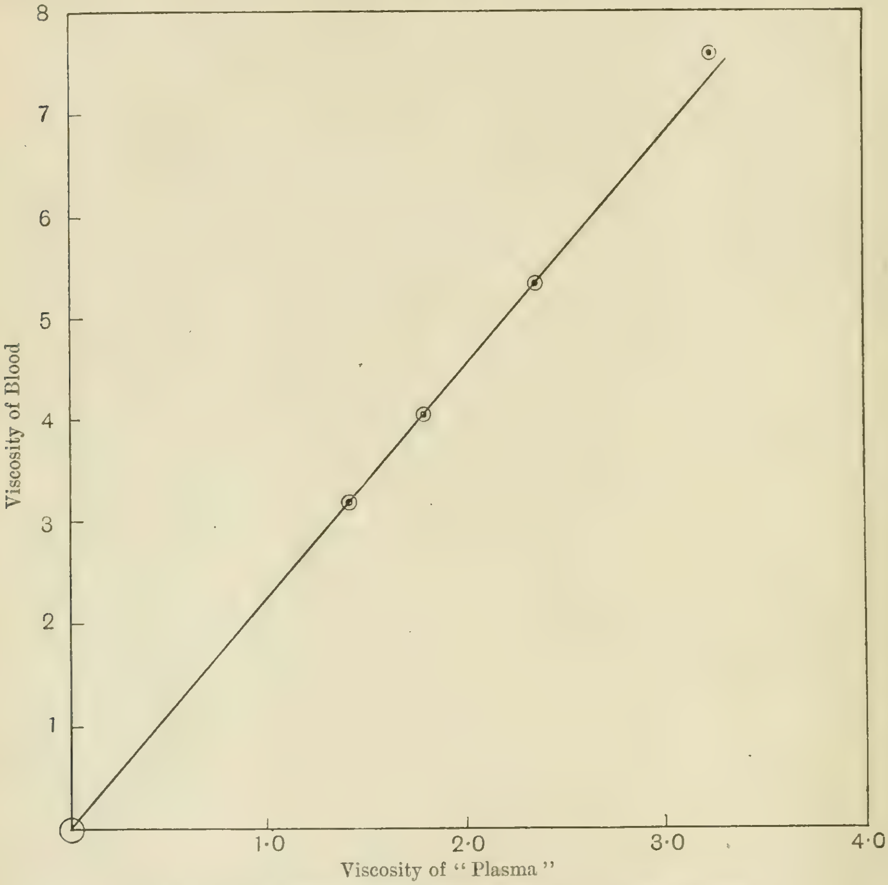

Fig. 2. Graph of relationship of viscosity of blood to that of plasma. The observed points are seen to lie along a straight line.

VI. THE CONTRIBUTION OF THE CORPUSCULAR VOLUME TO THE VISCOSITY.

The variation in the viscosity of the blood with the volume of the corpuscles present is shown in Table VII. Blood from various sources (indicated) was used, the viscosity of whole blood and plasma and the total volume of the corpuscles present were measured as before described.

TABLE VII.

1 Source of corpuscles	2 Nature of "plasma"	3 Viscosity of "blood" comp. with water	4 Viscosity of "plasma" comp. with water	5 Viscosity of "blood" comp. with "plasma"	6 % corpuscular volume calculated from Formula 1	7 % corpuscular volume found
Sheep	Saline	30·50	1·00	30·50	91·0	90·5
,,	,,	15·05	1·00	15·05	78·9	79·1
,,	,,	11·13	1·00	11·13	74·5	73·5
,,	,,	7·10	1·00	7·10	63·5	62·5
Dog	Plasma	(19·6)*	(3·00)*	6·53	60·8	59·3
,,	,,	9·38	1·56	6·01	58·0	58·3
,,	,,	7·75	1·56	4·97	51·0	52·1
Cat	,,	8·04	1·62	4·96	50·05	51·5
Dog	,,	6·90	1·56	4·42	46·2	47·0
,,	,,	5·42	1·75	3·10	31·2	36·3
Human	Plasma and citrate	3·00	1·31	2·29	18·2	23·5
,,	,, ,,	2·30	1·31	1·76	7·9	11·75
,,	,, ,,	1·55	1·31	1·19	4·17	5·88

* Times of flow. Viscosimeter broken before calibration.

In column 7 of this table are given the haematocrit volumes and in column 6 a series of haematocrit volumes calculated from the formula

$$\frac{100}{P} = \left(\frac{n}{n-1}\right)^3 \quad \text{..(1)}$$

where P = volume of corpuscles as % of total volume of blood.

n = the ratio of the viscosity of the whole blood to that of the plasma.

The agreement above a corpuscular volume of 45 % is within ± 1 % which is near the limit of error of the measurements. The curve *ACD* in Fig. 3 is the curve of the above equation and shows the agreement graphically.

The formula is one established mathematically by Hatschek [1912] for emulsions, when the volume of disperse phase is over 60 % and the rate of shear sufficient to convert the droplets of disperse phase into flat dodecahedra. The viscosity of the disperse phase does not appear in the formula, the only essential being that the disperse phase is easily deformable. This condition is fulfilled by the red corpuscles. The formula does not hold for emulsions below 60 % as at that point the droplets of disperse phase become spherical. In the case of blood corpuscles the discoid shape sufficiently approaches that of a dodecahedron for the formula to hold down to 45 %. Below this there are other factors, including the rotational energy of the corpuscles, which become increasingly greater as the corpuscles become spaced out, and this, in its turn, causes the calculated and observed values to diverge more and more. The observed values below 40 % lie about a straight line *AB* which is the graph of

$$n = 1 + \frac{KP}{100} \qquad \text{(2)}$$

where n and P have the values previously assigned and K is an arbitrary constant = approximately 6·3.

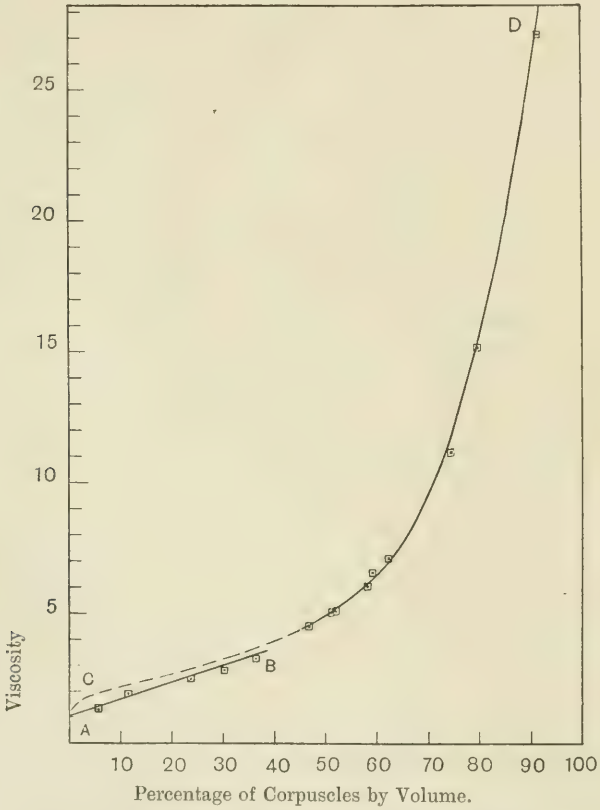

Fig. 3. Relation of viscosity to corpuscular volume.
⊡ observed points.
ACD = graph of formula 1.
AB = graph of formula 2.

This formula is similar to that deduced mathematically for suspensions of spheres by Hatschek [1912]

$$n = 1 + \frac{4{\cdot}5P}{100} \qquad \text{(3)}$$

but differs in the constant K which, as Hatschek pointed out, depends on the shape of the suspended body. 6·3 was selected as most nearly representing the observed figures. The calculation of the constant for a body of the shape of the corpuscle presents considerable difficulty.

VII. Discussion.

1. The relationships established above will perhaps be made clear by the following. In narrow passages the blood corpuscles when crowded together move edge foremost. Their distribution next to the wall of the capillary can be represented by the diagram in Fig. 4. The layer of corpuscles next the wall (A) is practically stationary. That next, at B, is travelling slowly, that at C a little faster and so on. The internal friction is produced between the layers of plasma and the surface of the corpuscles: the total kinetic energy of the corpuscles is the same at the beginning and the end of the capillary tube so that the energy degraded into heat in the tube is due entirely to the internal friction of the plasma moving relatively to the corpuscles. This being the case, the conditions can be made clearer by treating the corpuscles as fixed and the plasma as moving along the chinks between them with varying velocities, slowest near the wall and greatest in the centre of the tube. The rate of flow of fluids along such narrow passages was shown by Poiseuille to be inversely proportional to the viscosity. Hence the influence of the plasma on the viscosity of the blood is explained.

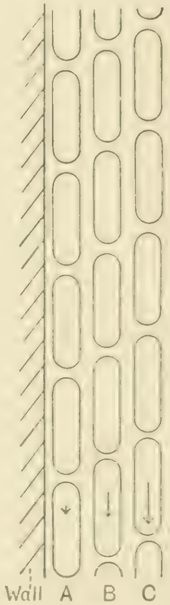

Fig. 4. Diagram of the flow of corpuscles along the wall of the capillary viscosimeter. The arrows represent by their direction and length the direction and velocity of each row of corpuscles.

He also showed that the rate of flow of fluids along narrow capillary passages is proportional to the fourth power of the radius of the capillary. This explains why the increase of the volume of the corpuscles present, which narrows the passages along which the blood flows, produces such a markedly increasing influence on the viscosity of the blood.

2. These experiments suggest a few conjectures as to the shape-consistency of the red corpuscles. Any evidence available goes to show that other living cells are more or less fluid and tend therefore to assume a spherical shape when freed from constraining influences, or unless they are

definitely amoeboid; *e.g.* in the numerous cultural experiments with living tissue cells [N. C. Lake, 1916] the growing cells which are found in such cultures are nearly always spherical whatever their origin, unless they become adherent to the lymph or other solid culture medium used. But the red corpuscles, although still undergoing metabolic changes (consumption of O_2, glucose [see MacLean 1916] *et alia*) are flat discs and therefore rigid, if elastic. Even the nucleated corpuscles of birds etc. are flattened. Now consider for a moment what would happen if the red corpuscle were a sphere of liquid protoplasm such as the white corpuscle. If the total volume of corpuscles present were less than 60 % the spheres would be completely separated by plasma, but at 60 % the corpuscular volume would be so great as to cause the spheres to come in contact. Up to that point the viscosity would increase almost in a straight line relationship with the increase in volume, but above it two things may happen depending on the velocity of flow. If the velocity of flow is insufficient to deform the spheres into flat discs the corpuscles would "jam" and cease to flow through the capillaries or only flow exceedingly slowly. Plasma would still flow through the interspaces. Such a condition as this however is much more rarely set up with disc-shaped corpuscles.

3. The white corpuscles have been included in the total volume of corpuscles measured in the above experiments. The rate of shear in the capillary viscosimeters used is probably sufficient to produce in them the requisite change of shape, but in any case their relative volume in most cases is so small as not to affect the results appreciably. In conditions such as myelocytic leukaemia, however, the relative volume of the white corpuscles is so large that considerable modifications of the conditions of capillary flow must arise, and it was hoped, but in vain, that this question of the influence of white cells on the viscosity might have been investigated before publishing these figures. It will at once occur to the reader that the amoeboid properties of the white cell would prevent the block in the capillaries becoming absolute, and that therefore the circulation of dead and of living leucocytes would produce different results.

4. These experiments suggest one or two observations as to the microscopic vascular changes associated with inflammation and infarction. The first stage in the vascular change accompanying inflammation is a dilatation of the capillaries accompanied by an increased rate of flow: this is succeeded by an increased permeability of the capillary wall leading to an increased outpouring of lymph: the rate of flow of the blood then gradually slows down

and finally almost ceases. It is suggested that this stage of stasis in inflammatory conditions is due not to any special change in the wall but to the very great and increasing rise in the viscosity of the blood consequent on the loss of plasma. This condition of stasis would be commoner than it is were the corpuscles not flattened discs.

SUMMARY.

1. A form of capillary viscosimeter for blood is described in which stirring during the observations is possible.

2. Errors and precautions during the measurement are discussed.

3. The viscosity of the blood is shown to be a constant multiple of that of the plasma for the same number of corpuscles.

4. Mathematical relationships are shown to hold between the number of corpuscles and the viscosity.

5. Some of the bearings of the results are discussed.

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I have to thank Professor Bainbridge for much kind encouragement and criticism during the progress of the work.

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V. ON β-CHOLESTANOL, SOME OF ITS DERIVATIVES AND OXIDATION PRODUCTS.

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Gardner and Godden [1913] showed that coprostanone on oxidation by chromic acid yielded a small quantity of an acid, melting at 247°, identical with that obtained as a bye-product in the preparation of coprostanone from coprosterol. On oxidation by ammonium persulphate the coprostanone yielded as main products two isomeric substances of the formula $C_{27}H_{46}O_2$, melting respectively at 183–184° and 157–158°. These appeared to be lactones of a hydroxyacid or hydroxyacids of the formula $C_{27}H_{48}O_3$. It seemed possible that light might be thrown on the relationship of coprosterol to the various isomeric artificial bihydrocholesterols—ψ-coprosterol, β-cholestanol, etc.—by a study of their oxidation by means of ammonium persulphate. We therefore commenced our experiments with β-cholestanol but the work has been much delayed by the absence of one of us on more urgent duties.

In the meantime Windaus and his co-workers have very materially increased our knowledge of this group of alcohols. Uibrig and Windaus [1913, 1914, 1915] working on the four alcohols, (1) β-cholestanol, (2) ϵ-cholestanol, (3) coprosterol, and (4) ψ-coprosterol, showed that β-cholestanol and ϵ-cholestanol can be changed into one another by boiling in amyl alcohol solution with sodium amylate, and differ from one another only in the stereo-position of the hydroxyl group with regard to an asymmetric carbon atom, as in the case, for instance, of borneol and iso-borneol. A similar relationship obtains between coprosterol and ψ-coprosterol, as had been previously pointed out by Dorée and Gardner [1908]. β-Cholestanol and coprosterol, they maintain, differ from one another in the stereo-position of a hydrogen atom with regard to another asymmetric carbon atom, but they had not been able to change the one into the other. The relationship of ϵ-cholestanol to ψ-coprosterol is similar.

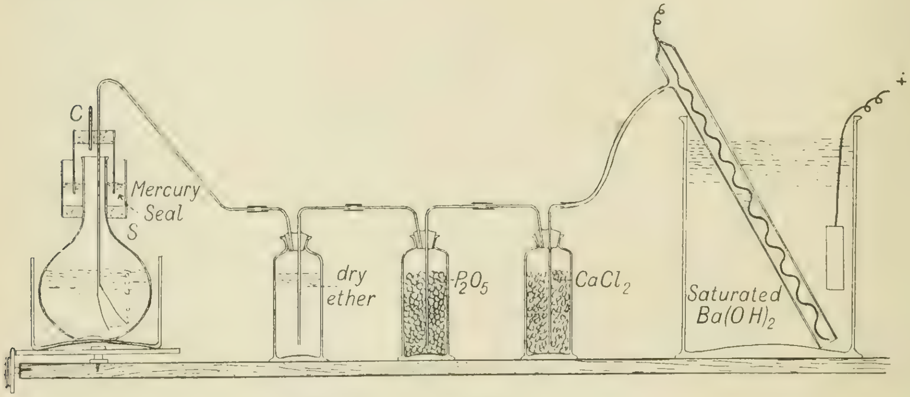
In a later paper Windaus [1916] gave an account of the reduction of cholesterol by means of hydrogen at 200° in the presence of reduced nickel and described a new alcohol, γ-cholestanol, M.P. 146° and $[\alpha]_D^{18} + 29{\cdot}9°$. This he found to be really a conglomerate of β-cholestanol, ψ-coprosterol and ε-cholestanol in the sense of the partial racemate compounded of β-cholestanol and ψ-coprosterol. They crystallise together like a single compound and the product, γ-cholestanol, has a similar melting point and similar specific rotary power to β-cholestanol, the only difference being that γ-cholestanol crystallises from dilute alcohol in the anhydrous condition, whereas β-cholestanol crystallises with one molecule of water. The separation of the constituents of γ-cholestanol was effected by taking advantage of the fact that, whereas β-cholestanol and coprosterol are precipitable by digitonin, the isomers ψ-coprosterol and ε-cholestanol do not form insoluble compounds with this substance. The β-cholestanol was removed by means of digitonin and the isomerides partially separated by crystallisation from methyl alcohol. ε-Cholestanol separates first and the part which remains in the crude ψ-coprosterol is re-arranged into β-cholestanol by boiling the mixture with sodium and xylene. This β-cholestanol was again precipitated by digitonin and the pure ψ-coprosterol obtained. Finally, Windaus found that the ψ-coprosterol could be partly transformed into the isomeric coprosterol by heating with alcoholic sodium ethoxide at 180°. The coprosterol was separated from the mixture by precipitating with digitonin and recovered from the digitonide in the usual manner by boiling with xylene. From the provisional formulae proposed by Windaus we should expect the oxidation products of β-cholestanol and β-cholestanone to be very similar, if not identical, with those obtained by Godden and Gardner from coprosterol and coprostanone. The dicarboxylic acid, melting at 247°, obtained from coprosterol by Godden and Gardner and by J. Adamla [1911] from γ-cholestanol, we did not find among the oxidation products of either β-cholestanol or β-cholestanone.

The β-cholestanone was less readily attacked by ammonium persulphate than coprostanone and the products obtained, though apparently lactones of the formula $C_{27}H_{46}O_2$, were different from those prepared from coprostanone and were more easily hydrolysed. They were very difficult to separate and the behaviour of the lower melting isomers suggested either that different isomers possessed the property of crystallising together like single substances, as Windaus has shown for different bihydrocholesterols, or that different isomers are readily re-arranged one into the other. None of the substances obtained was, however, identical with any of those got from coprosterol.

EXPERIMENTAL.

Preparation of β-cholestanol. The reduction of cholesterol to β-cholestanol was first carried out by Willstätter and Meyer [1908] by the use of hydrogen and platinum black in ethereal suspension. The exact technique of this method has been somewhat modified by us and with such good results that it may therefore be described. The dry pure ether used was prepared from the commercial product by treatment in the cold with about 10 % of its weight of a finely powdered mixture of dry potassium hydroxide and potassium permanganate in the proportion of about four parts of potash to one of permanganate. The mixture was occasionally shaken for a period of 24–28 hours and after settling the ether was decanted and distilled over phosphorus pentoxide.

The hydrogen, which it is essential should be pure and dry, was prepared electrolytically, the electrolyte employed being a saturated solution of barium hydroxide. The cathode was a silver coil and the anode a platinum coil. The general arrangement of the voltameter is shown in the accompanying diagram.



The Preparation of Dihydrocholesterol.

The hydrogen passed through a calcium chloride tower, through phosphorus pentoxide and then through dry ether. The latter served the purpose of arresting any traces of phosphorus pentoxide and of making up for loss of ether from the cholesterol solution by its own evaporation. The ethereal solution of cholesterol, which should be not more than three-fourths saturated, contained about 8 grams of platinum black to 400 cc. of the solution. The platinum black was prepared according to the method of Loew [1890].

It was found very necessary to keep the solution of cholesterol continually and vigorously shaken, in order that the heavy platinum black should be in a continual state of suspension. We believe this suspension can be more completely established by the method shown in the figure. The flask containing the solution is kept in rapid rotation; the stirrer is fixed, but is composed of a bent, wide-mouthed tube, so arranged as to stir up the rotating ether. Such a movement can be readily obtained by mounting a flask in a glass dish by means of four corks and mounting the dish on a horizontal wheel rapidly rotated by means of a pulley coupled with a motor. Such a device was found completely to reduce 50 grams of cholesterol in 24 hours. The evaporation of the ether is avoided largely by the introduction of the ether wash bottle, reducing the outlet of the flask by the use of a mercury valve *S* and allowing the gas to escape by a capillary tube *C*. The completion of reduction is best ascertained by an estimation of the specific rotary power which is the reverse of that of cholesterol.

The rotation was found to be $[\alpha]_D^{18} + 28{\cdot}8^\circ$, a value agreeing with that given by Willstätter and Meyer.

A number of esters were prepared at the beginning of our work with a view of comparing their properties with those of other isomers; we give here the properties of the chloroacetate, benzoate, and stearate which as far as we are aware do not appear to have been fully described.

β-Cholestanol chloroacetate. Two grams of β-cholestanol were dissolved in 20 cc. chloroform which had been previously freed from alcohol by washing, and then dried, and distilled. One gram of chloroacetyl chloride was added and the mixture gently boiled under a reflux for a few minutes. Another gram of the reagent was then added and the boiling continued for 30 minutes. The solution was then evaporated and the residue taken up in ethyl acetate, from which, on cooling, crystals were readily obtained. They were purified by recrystallisation from a mixture of chloroform and ethyl acetate. The yield was approximately theoretical. β-Cholestanol chloroacetate crystallises in glistening plates not unlike cholesterol in appearance and melts at 178–179°. It is readily soluble in ether and in chloroform but only slightly so in cold ethyl acetate.

Analysis: 0·2321 g.; 0·2210 H_2O; 0·6361 CO_2.

Found C = 74·15, H = 10·58.

Calculated for $C_{29}H_{49}ClO_2$ C = 74·92, H = 10·54.

β-Cholestanol benzoate. 1·5 grams of β-cholestanol were dissolved in 15 cc. of pure dry pyridine and 2·5 cc. of benzoyl chloride added. The mixture was allowed to stand overnight, poured into about 50 cc. of water and the precipitated benzoate filtered off and washed. It was crystallised from a mixture of benzene and acetone and finally from ethyl acetate and was thus obtained in the form of small plates. The yield was satisfactory. It is only slightly soluble in cold alcohol and not very soluble in hot. It is fairly soluble in ether, readily in benzene, and slightly so in acetone. It melts at 155° and during the melting process displays an extraordinarily brilliant fluorescence. On gently heating in a melting point tube it assumes at about 138–139° a reddish tinge, and at 140° begins to soften and run together to an opaque opalescent mass of a red tinge with flashes of green. At 145° it is still opaque and the play of colours becomes more intense, red and emerald green predominating according to the point of view. The fluorescence becomes brighter as the temperature rises, until at 155° the colours suddenly vanish and the liquid becomes clear. On cooling, the liquid becomes opaque again at 155° and the colour display appears again in the reverse order. The colours are not shown in transmitted light. The colour display may be very strikingly demonstrated by heating between two microscope slides. Owing to this colour display the exact melting point could not be determined.

In chloroform solution the rotation was found to be $[\alpha]^{23}_{D} + 23{\cdot}3°$.

Analysis: 0·2222 g.; 0·21 H_2O; 0·6725 CO_2.

Found C = 82·54, H = 10·50.

Calculated for $C_{34}H_{52}O_2$, C = 82·92, H = 10·57.

β-Cholestanol stearate. An attempt to obtain this substance by a process similar to that employed for the preparation of the benzoate was not successful, only a very small percentage yield being obtained. A good yield was obtained by adding to 1 gram of the β-cholestanol about twice its weight of stearyl chloride, the whole being in chloroform solution. The solution was left to stand in a closed flask for a day, then boiled gently for half-an-hour and finally evaporated to dryness. This stearate was almost insoluble in alcohol, but soluble in a mixture of alcohol and chloroform, from which it readily crystallised in soft flaky crystals. It melts at 100° and is readily soluble in ether. The rotation in ether solution was $[\alpha]^{20}_{D} + 18{\cdot}4°$.

Analysis: 0·1599 g.; 0·1869 H_2O; 0·4856 CO_2.

Found C = 82·82, H = 12·99.

Calculated for $C_{45}H_{82}O_2$, C = 82·57, H = 12·54.

Oxidation of β-cholestanol. Diels and Abderhalden [1906] first showed that β-cholestanol is readily oxidised to a ketone, β-cholestanone, by the action of chromic acid in acetic solution. This ketone melts at 128–129°, whereas coprostanone melts at 62–63°. In the preparation of coprostanone from coprosterol Godden and Gardner found that the yield never exceeded

70 %, and the ketone was accompanied by considerable quantities of a dibasic acid, $C_{27}H_{16}O_4$ melting at 247°, and smaller amounts of a non-crystalline acid.

In our experiments on the preparation of β-cholestanone the yield was from 80–90 %. The glacial acetic solution of the oxidation product was diluted with water and extracted with ether. On shaking the washed ethereal solution of the ketone with dilute aqueous potash a trace of a crystalline acid was obtained on acidifying the alkaline extract. The amount of this acid appeared to be slightly increased by conducting the oxidation in more gentle fashion. We did not obtain it in sufficient quantity for further investigation, but it was quite different from the above mentioned dibasic acid, M.P. 247°, obtained from coprosterol.

Oxidation of β-cholestanone by ammonium persulphate. This oxidation was carried out in a manner somewhat similar to that described by Godden and Gardner in the case of coprostanone, but took place less readily. Four grams of the ketone were dissolved in 150 cc. of glacial acetic acid and 4 grams of ammonium persulphate in 8 cc. of water were added. The mixture was placed on a water bath for an hour and left over-night at a temperature of about 80°. The next day another 4 grams of the persulphate were added in small amounts and the mixture allowed to stand for 4 days at 80°. On cooling crystals separated and were filtered off. The filtrate was then diluted and extracted with ether. This extract was added to an ethereal solution of the crystals already separated and the whole extracted by shaking with a solution of sodium carbonate. This extract on acidification yielded a trace only of an oil. The ethereal solution was then further extracted with 10 cc. potash. The ether solution thus extracted was found to contain a small amount of unchanged ketone. Several oxidations were carried out in this way. The potash extract on acidification with hydrochloric acid yielded a flocculent precipitate, soluble in alcohol, from which it crystallised in clots of fine needles. These were recrystallised from ethyl acetate and melted at about 180°. On dissolving the crystals in ether and extracting with potash it was found that now very little of the substance was removed. On evaporating the ether and recrystallising the residue from alcohol crystals melting round 180° were again obtained. This behaviour recalls the properties of the lactones from coprostanone described by Godden and Gardner. The crystals were readily soluble in ether, alcohol, or ethyl acetate, and when allowed to cool after melting in a capillary tube showed an opalescent appearance and after 30 seconds the contents of the tube suddenly gave a slightly audible crackle. This phenomenon, by remelting, could be repeated indefinitely.

This crystalline matter was fractionally crystallised from various solvents —alcohol, acetone, ether and methyl alcohol. A fraction separated from alcohol in clots of microscopic needles. This, after repeated recrystallisation from alcohol and from acetone melted sharply at 201–202°.

Analysis: 0·1034 g.; 0·10655 H_2O; 0·30585 CO_2.
Found C = 80·67, H = 11·45.
Calculated for $C_{27}H_{46}O_2$, C = 80·52, H = 11·50.

It was not attacked by acetic anhydride and had no ketonic properties. It was not hydrolysed by shaking the ethereal solution with potash, strong or dilute, but on boiling with alcoholic soda it yielded a salt soluble in water. On acidification the substance was re-formed and after recrystallisation from alcohol melted at 201–202°. It was presumably therefore a lactone of an acid $C_{27}H_{48}O_3$.

In addition to this lactone a small quantity of a body melting at 184–186° was obtained, the solubility of which was of the same order. This crystallised from alcohol in thin plates or needles very similar in appearance to the substance of the same melting point obtained by Godden and Gardner from coprostanone. It was not, however, identical, as on mixing the two substances the product melted between 160° and 170°. The amount of the pure substance was too small for combustion or further examination.

The crystalline matter left after the separation of these two bodies consisted of small needles and had the appearance of a single substance. Its behaviour on melting however showed that this was not the case, as it gradually softened through a considerable range of temperature. After repeated fractionation the following fractions were obtained:

A. A very small fraction melting at 98–100°. On combustion this was found to contain 80·5 % of carbon and 12·2 % of hydrogen. It may possibly be a low-melting lactone $C_{27}H_{46}O_2$, but the quantity was too small for further investigation.

B. Fine matted needles, which on heating began to shrink together at 144°, softened and melted at 152–153° and ran to a clear liquid at 157–158°.

C. Indeterminate crystalline matter. On heating it began to shrink together at 120° and melted at 130° to a turbid liquid, which became clear at 138–139°. This behaviour was shown again on cooling and re-heating.

D. Small needle-like crystals. It began to soften at 143°, softened at 150° and ran between 159° and 161° to a clear liquid. On cooling long needles began to form in the clear liquid at 158–159°, but the whole mass did not become opaque until a much lower temperature. On re-heating it became clear again at 150°, but the needles did not vanish until 159–161°. Further recrystallisation did not alter the melting point materially.

Analysis:

(1) 0·1005 g.; 0·1013 H_2O; 0·2946 CO_2.

(2) 0·0894 g.; 0·0915 H_2O; 0·2633 CO_2.

		C	H
Found	(1)	79·94	11·20
	(2)	80·32	11·50
Calculated for $C_{27}H_{46}O_2$,		80·52	11·50.

This was the main fraction. It was different in properties from the substance of similar melting point obtained from coprostanone. On boiling with weak alcoholic soda it yielded a sodium salt which dissolved in water to a soapy solution. On acidification the substance was not re-formed again, but a pasty precipitate was obtained. We had not sufficient for further examination of this acid. The substance was evidently not pure, but consisted mainly of a lactone or mixture of lactones, of the above formula.

Fraction C was analysed.

0·1119 g.; 0·1134 H_2O; 0·3366 CO_2.

Found C = 82·04, H = 11·26.

It was thought that perhaps this might contain some unchanged ketone, or even unchanged β-cholestanol, but mixed meltings did not seem to bear this out.

Fraction B was also a mixture and on combustion was found to contain 81·7 % of carbon and 11·44 % of hydrogen.

It was thought that if these intermediate fractions consisted of mixtures of high and low melting lactones it might be possible to destroy the lower melting ones by further oxidation with chromic acid, as Godden and Gardner had found that this was the case with similar mixtures from coprostanone. The results were negative and crop C gave a product of more or less unchanged melting point.

It would appear from these results that β-cholestanone on oxidation with persulphate yields a mixture of lactones of the formula $C_{27}H_{46}O_2$, probably two of high melting point and one or more of lower melting point, but as far as we were able to ascertain they were different from those obtained from coprostanone.

We hope later on to be able to give a more complete account of these various substances.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society for assistance in carrying out this work.

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VI. SUR LA FERMENTATION DE L'ACIDE GLYOXYLIQUE.

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EN étudiant l'action de la levure, préparée d'après ma méthode, sur quelques acides organiques, j'ai fait aussi des expériences sur l'acide glyoxylique. Celui-ci, ayant une fonction d'aldéhyde, présente sûrement un intérêt particulier. L'action de la levure vivante sur l'acide glyoxylique a été examiné par Neuberg et Tir [1911], mais le résultat a été négatif: de toutes les races employées par ces auteurs une seulement (la race A) a donné un peu plus de CO_2 que le témoin (7 cc. de CO_2 contre 1·5 cc.) dans 2 tubes de Schrotter.

D'après mes expériences il faut admettre que la levure de Lebedev peut décomposer l'acide glyoxylique avec dégagement de CO_2. Il n'est pas facile pour le moment d'expliquer de quelle manière agit cette réaction curieuse. A peine pourrait-on supposer que le dégagement de CO_2 soit dû tout simplement à l'excitation de l'autofermentation, parce que le même phénomène a lieu lorsqu'on emploie du suc de macération. Parallèlement au dégagement de CO_2 il se forme une quantité notable de CH_3COH. Si l'on se souvient de ce qu'on peut obtenir l'acide glyoxylique par l'oxydation de C_2H_6O, ce ne serait peut-être pas trop osé d'admettre que la levure puisse réduire celui-là en le transformant en C_2H_6O, étant donné que l'aldéhyde acétique lui-même se transforme en partie sous l'action de la levure en alcool éthylique.

Exp. 1. On a pris 6 fioles d'Erlenmeyer avec une soupape de fermentation de Meissl (Nos. 1–6). Elles ont été chargées de 1 gr. de levure + 50 cc. d'eau + 0·2 cc. de toluène. Aux Nos. 1–4 on a ajouté 100 mg. d'acide glyoxylique à chacune.

Température 25°.

t.	No. 1	No. 2	No. 3	No. 4	Moyen	Moyen des Nos. 5 et 6
Après 17 h. (mg. de CO_2)	112	114·5	104·5	115	111·5	85·5
,, 45 ,, ,,	145	150·5	134·5	159	147·0	114·5
,, 65 ,, ,,	154	161·0	142·0	178	159·0	123

Alors on obtient par soustraction: 111·5 – 85·5 = 26 mg.; 147 – 114·5 = 32·5 mg.; 159 – 123 = 36 mg. de CO_2 pour chaque fiole.

Exp. 2 est analogue à la précédente.

t.	No. 1	No. 2	No. 3	No. 4	Moyen	No. 5	No. 6	Moyen
4 h.	20·5	18·5	27	19·0	21·0	24	16	20·0
8 h.	66·5	72·5	72	71·5	70·5	50	39	44·5
24 h.	101·0	104·5	100	101·5	102·0	84	63	73·5

21 – 20 = 1 mg.; 70·5 – 44·5 = 26 mg.; 102 – 73·5 = 28·5 mg. de CO_2 pour chaque fiole.

Dans les Nos. 3 et 4 et dans les témoins (Nos. 5 et 6) on a dosé CH_3COH en forme de sa *p*-nitrophénylhydrazone sans la recristalliser (F. 127°). On a mélangé le contenu des Nos. 3 et 4 et des Nos. 5 et 6; de cette manière on a obtenu 2 portions de liquide; on les a neutralisées soigneusement et on en a fait distiller $\frac{1}{3}$ de volume, en refroidissant le récipient avec de la glace fondante. Dans ces conditions il ne passe que des traces d'acide glyoxylique comme cela a été prouvé par une expérience préliminaire. On a obtenu 35 mg. de hydrazone (F. 127°); le témoin au contraire n'en a rien donné.

Exp. 3. On a fait fermenter l'acide glyoxylique avec du sucre (pour 100 mg. d'acide 200 mg. de sucre) et on a dosé CH_3COH. T = 25°.

t.	ac. glyox. + sucre		ac. glyoxylique		sucre		témoins	
12 h.	147	156	87	—	136	134	78·5	69·5
33 h.	174	178	108	—	166	178	97·5	88·0
47 h.	209	208	130·5	134	190	218	118·5	107·0
	32 mg. d'hydrazone		30 mg. d'hydrazone		8 mg. d'hydrazone		traces	

Exp. 4. On a pris 4 fioles et on les a chargées chacune de 40 cc. de suc de macération; 2 fioles ont été additionnées chacune de 62·5 mg. d'acide. On a ajouté, comme toujours, à chaque fiole du toluène. T = 25°.

t.	ac. glyoxylique		témoins	
21 h.	9	12·5	4	4·6
45 h.	19	22·5	7	6·5
69 h.	27	27·5	8	8·0

Le suc qui a été chauffé pendant 10 min. à 70° n'agissait pas sur l'acide glyoxylique.

Exp. 5. On a pris 4 fioles avec des soupapes de fermentation; à chacune d'elles on a ajouté 22·5 cc. de suc. Aux 2 fioles on a ajouté en outre 2·5 cc. de solution à 3·54 % d'acide glyoxylique à chacune (88·5 mg.).

t.	CO_2 (ac. glyoxylique)		CO_2 (témoins)	
48	13·5	16	6	6
72	20·5	25	11	9

29·5 – 12 = 17·5 mg.; 45·5 – 20 = 25·5 mg. Pour une fiole: 9 et 13 mg. de CO_2.

Exp. 6. 4 fioles ont été chargées de la levure qu'on a conservée au laboratoire au moins pendant une année. Pour 10 gr. de levure on a pris 50 cc. d'eau. Aux 2 fioles on a ajouté en outre 100 mg. d'acide glyoxylique à chacune. T = 25°.

t.	CO_2 (ac. glyoxylique)		CO_2 (témoins)	
11 h.	48·5	50·5	50·0	—
24 h.	100·5	105·5	98·5	105
29 h.	113·5	118·0	114·5	119

Si l'on considère la quantité de CO_2 dégagé, on ne s'aperçoit pas d'une différence quelconque entre les fioles chargées de CHO . CO_2H et les témoins, néanmoins on a obtenu 28 mg. d'hydrazone qui, recristallisée une fois, avait le point de fusion 128°. Le témoin n'a donné après la distillation aucune hydrazone. Ce fait curieux dit en faveur de la supposition que l'aldéhyde acétique se forme à cause de la réduction de l'acide glyoxylique. Pour faire ressortir plus clairement l'influence de la concentration, j'ai fait l'expérience suivante.

Exp. 7. On a pris 5 fioles (Nos. 1–5) avec des soupapes de fermentation; on a ajouté 10 gr. de levure, 50 cc. d'eau et 0·2 cc. de toluène à chacune. Aux fioles Nos. 1, 2 et 3 on a ajouté en outre 300, 200 et 100 mg. d'acide glyoxylique. T = 25°.

t.	300 mg. d'ac. glyox.	200 mg. d'ac. glyox.	100 mg. d'ac. glyox.	témoins	
21 h.	19	40	73	65	33·5
27 h.	30	70	109	94	69·5
39 h.	40	84	137	105	99·5
64 h.	40	84	144	120	121·0

Le mécanisme de la décomposition n'étant pas bien clair, je me suis posé la question si l'acidité du milieu influencerait l'autofermentation d'une manière sensible.

Exp. 8. 2 fioles ont été chargées de 10 gr. de levure, 50 cc. d'eau et 0·2 cc. de toluène. A une d'elles on a ajouté 0·1 cc. de HCl.

t.	HCl	témoin	différence
3 h.	50 mg. de CO_2	21 mg. de CO_2	29 mg. de CO_2
20 h.	112 ,, ,,	63 ,, ,,	49 ,, ,,
28 h.	126 ,, ,,	72 ,, ,,	54 ,, ,,
55 h.	156 ,, ,,	113 ,, ,,	43 ,, ,,
70 h.	191 ,, ,,	166 ,, ,,	25 ,, ,,

Ainsi devient-il plausible que l'acide minéral puisse favoriser l'autofermentation de la levure, il faut donc s'attendre à ce que les acides organiques puissent produire, suivant le degré de leur dissociation électrolytique, le même effet.

Les expériences avec les acides glycolique, acétique, lactique, etc. m'ont donné un résultat nettement positif. J'en ferai connaître quelques-unes faites à ce sujet.

Exp. 9. 4 fioles ont été chargées chacune de 10 gr. de levure et 50 cc. d'eau. Aux 2 fioles on a ajouté 200 et 100 mg. d'acide glycolique.

t.	200 mg.	100 mg.	témoins	
17 h.	70	76	66·5	59·0
24 h.	94	95	80·5	77·5
38 h.	130	139	125·5	124·5
43 h.	146	168	154·0	153·0

Exp. 10. Au lieu de 10 gr. de levure on a pris 25 cc. de suc de macération. Aux 2 fioles on a ajouté 62 mg. d'acide glycolique à chacune.

t.	ac. glycol.		témoins		différence pour 1 fiole
14 h.	9	12	4	4·0	6 mg. de CO_2
45 h.	19	22	7	6·5	14 „ „
69 h.	27	28	8	8·0	20 „ „

Cette expérience dit plutôt en faveur de la supposition que l'acide glycolique soit décomposé sous l'influence de la levure. Je n'ai pas réussi à préciser définitivement quelle était l'action de HCl sur le suc de levure, parce qu'une petite quantité d'acide minéral suffisait déjà pour en faire une coagulation partielle.

Exp. 11. On a pris 6 fioles chargées chacune de 10 gr. de levure + 50 cc. d'eau + 0·2 cc. de toluène (Nos. 1–6). Aux fioles 1 et 2 on a ajouté 378 mg. d'acide glycérique, aux fioles 3 et 4 209 mg. d'acide acétique à chacune.

t.	ac. glycér.		ac. acét.		témoins		différence pour 1 fiole	
							ac. glycér.	ac. acét.
28 h.	166	183	117	116	66	67	108 mg. de CO_2	50 mg. de CO_2
55 h.	188	166	166	158	102	112	70 „ „	55 „ „

Exp. 12. Cette expérience est analogue aux précedentes; aux 2 fioles on a ajouté 200 mg. d'acide lactique à chacune.

t.	ac. lactique		témoins		différence pour 1 fiole
28 h.	109	121	66	61	51·5 mg. de CO_2
49 h.	132	150	104	91	43·5 „ „
72 h.	139	160	138	118	21·5 „ „

Elle montre que la différence diminue avec le temps, ce qui donne tout lieu à croire que le dégagement de CO_2 en présence de l'acide lactique était dû plutôt à l'action stimulante exercée par lui qu'à la décomposition de l'acide lactique, d'autre part les expériences que j'ai publiées antérieurement ont démontré [Lebedev, 1915, 1916] que la présence de l'acide lactique se manifestait seulement par l'augmentation de la quantité de CO_2 dégagé, mais pas d'alcool formé; le rapport $\frac{CO_2}{C_2H_6O}$ pendant l'autofermentation reste au contraire presque égal à l'unité. On voit de tout ce que je viens de dire qu'il n'est pas facile de se faire une idée précise de ce qui se passe en vérité et il faudra encore beaucoup d'expériences pour y mettre une pleine clarté. La fermentation de l'acide glycérique a été établie par moi [Lebedev, 1914] fermement, parce que le dégagement de CO_2 était accompagné de la formation de CH_3COH. Dans le travail fait par moi[1] en collaboration avec M. Polonsky nous avons dosé tous les produits qui se formaient pendant la décomposition de l'acide glycérique, à savoir CO_2, $CH_3.COH$, $CH_3.CH_2OH$ et $CH_3.CO_2H$, et nous avons constaté que la somme ($CH_3.COH + CH_3.CO_2H + CH_3.CH_2OH$) était presque égale à la quantité de CO_2. En ce qui concerne les acides glyoxylique, acétique, lactique et glycolique la question reste ouverte, parce que, excepté CO_2 et $CH_3.COH$, on n'a pas découvert, tout au moins momentanément, d'autres produits de leur décomposition.

Il était intéressant de voir quelle action exerçait un milieu neutre sur le travail de la levure de Lebedev pendant la fermentation du sucre, c'est pourquoi j'ai fait quelques expériences en présence de $CaCO_3$; je vais reproduire ici une de celles-ci.

Exp. 13. 4 fioles chargées chacune de 10 gr. de levure + 50 cc. d'eau + 0·2 cc. de toluène. On a ajouté au No. 1—5 gr. de sucre, au No. 2—5 gr. de sucre + 1 gr. de $CaCO_3$, au No. 3—1 gr. de $CaCO_3$.

t.	1 sucre	2 sucre + $CaCO_3$	4 témoin	3 tém. + $CaCO_3$	No. 1 – No. 4	No. 2 – No. 3	(1 – 4) – (2 – 3)
24	1173	879	82	69	1091	810	281
27	1379	1048	156	119	1223	929	294
48	1447	1058	229	162	1218	896	322

On a dosé $CH_3.COH$ et on a obtenu: No. 1—5·5 mg., No. 2—10·5 mg. de *p*-nitrophénylhydrazone, Nos. 3 et 4—des traces de celle-ci.

On peut expliquer l'action de $CaCO_3$ de trois manières. 1. Le milieu neutre est nuisible à l'action de la zymase. 2. Le sel de Ca de l'éther phosphoré se décompose plus lentement que l'éther libre. 3. Le sel de Ca de l'acide pyruvique, si celui-ci vraiment est un produit intermédiaire, ne fermente pas si bien que l'acide libre et en outre, la marche de la réaction étant dérangée, l'aldéhyde acétique apparaît, ce qui concorde aussi avec des expériences de Fernbach et Schoen [1913, 1914]. Les expériences ultérieures montreront quelle des trois explications est la plus probable.

En ce qui concerne l'acide glyoxylique il faut noter ici seulement le fait de la formation de $CH_3.COH$ et par conséquent de $CH_3.CH_2OH$. S'il y a vraiment une simple réduction de l'acide, il faudrait admettre qu'il soit un accepteur énergique de H, parce que d'après la réaction

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[1] Ce travail paraîtra bientôt.

$$CHO.CO_2H + 8H = 2H_2O + CH_3.CH_2OH$$

une molécule de celui-ci pourrait joindre 8 atomes de H ou $\frac{1}{8}$ de son poids. Il est alors possible que la décomposition de $C_2H_2O_3$ soit déterminée par la même cause, puisque dans ce cas une partie des molécules pourrait s'oxyder aux dépens de la réduction de l'autre partie de la même molécule.

L'alcool qui se forme en l'absence de l'air dans les tissus végétaux ne résulte pas toujours de la fermentation du sucre. Sans parler de celui-ci, peut-être faudra-t-il ajouter aux sources connues depuis longtemps comme, par exemple, l'acide pyruvique (Neuberg) et glycérique (Lebedev), encore l'acide glyoxylique que l'on trouve si souvent dans les plantes. L'éclaircissement de cette question se rapportant entièrement au domaine de la physiologie végétale ne faisait pas l'objet de mes recherches.

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VII. SUR LA FORMATION DES ÉTHERS PHOSPHORÉS PENDANT LA FERMENTATION ALCOOLIQUE.

(*Communication préliminaire.*)

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Il y a quelques années j'ai démontré [1909, 1910, 1911] que pendant la fermentation du sucre l'éther phosphoré d'un hexose se formait. Dans le schéma que j'ai donné j'ai admis que préalablement il se formait un triose-phosphate. Cette supposition a été appuyée par le fait que la dioxyacétone donnait en fermentant un hexose-phosphate identique, évidemment par la condensation de l'éther phosphoré du triose [Lebedev, 1911, 2, 3; 1914; Lebedev et Griaznov, 1912]. En vue de l'importance de cette question, j'ai repris depuis une année une étude minutieuse pour voir si ce point de vue était juste. En modifiant la méthode dont je me suis servi auparavant pour l'isolement de l'éther et ayant pris une autre levure, j'ai pu, après un long et assidu travail, enfin constater d'une manière irréfutable la formation d'un éther ou plutôt d'un mélange des éthers phosphorés qui m'a donné les *p*-bromphénylhydrazone, *p*-bromphénylosazone et phénylosazone ayant un autre point de fusion et une autre composition que les combinaisons correspondantes, données par l'hexose-biphosphate. Les circonstances qui ne dépendent pas de moi m'ont obligé malheureusement d'interrompre pour une demi année l'étude commencée avec tant de succès; c'est pourquoi je me suis résolu de publier le résultat obtenu sans attendre l'accomplissement du travail en train ayant le but de jeter une lumière sur la décomposition primaire du hexose.

Méthode. 50 gr. de levure de Lebedev + 100 gr. de saccharose + 35 gr. de Na_2HPO_4 + 17 gr. de NaH_2PO_4 + 500 gr. d'eau + 1 cc. de toluène. Pour interrompre la fermentation, on ajoutait au moment voulu 2 ltr. d'alcool à 97°. On essorait le résidu à la trompe, on le lavait par de l'alcool dilué (1 : 1) et on le traitait par l'eau à plusieurs reprises; le filtrat (300 cc.) a été précipité par de l'alcool (1200 cc.). Après avoir laissé reposer le tout pendant 12 heures, on a transvasé l'alcool qui a été au dessus du résidu, on a redissous celui-ci dans l'eau et précipité par 3 volumes d'alcool. (La dernière fois on a précipité par 2 vol. d'alcool.) Par ce procédé on obtient 2 couches de liquide: la supérieure blanchâtre, opaque et très visqueuse, l'inférieure jaunâtre, presque transparente et moins visqueuse. Il était évident qu'il y avait au moins 2 substances différentes. Les deux couches ont été séparées à l'aide d'un entonnoir à robinet et chacune d'elles a été précipitée par l'acétate de plomb. Le résidu a été lavé avec une solution faible d'acétate de plomb et essoré à la trompe. La résidu a été délayé dans l'eau et traité par H_2S. Le filtrat a été traité avec de la phénylhydrazine à 100° ou avec une solution alcoolique de *p*-bromphénylhydrazine à 35°. On séchait les précipités après recristallisation dans l'appareil d'Abderhalden, modifié par moi [1911, 1, p. 257] à 37° et on les analysait. On a dosé le P par l'excellente méthode de Neumann.

Dans quelques expériences, avant de traiter avec de l'acétate de plomb, j'enlevais l'acide phosphorique au moyen du mélange magnésien. Sans décrire ici en détails toutes les expériences nombreuses que j'ai faites dans des conditions différentes, je n'en ferai connaître que quelques-unes, pour les autres je donnerai plus bas l'aperçu général des dosages des P et N des osazones recristallisées, obtenues ordinairement par l'action de la phénylhydrazine sur le mélange des deux couches. Il faut les envisager pour le moment comme des expériences d'orientation.

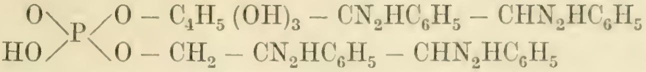
1. La fermentation allait 2 h. à 35°. L'osazone obtenue de la couche supérieure, recristallisée une fois de l'alcool à 85°. P—2·96 %. De grands faisceaux de très longues aiguilles d'une couleur orange. Le point de fusion 142°. L'osazone de la couche inférieure avait le point de fusion 142–144°. P—4.42 %.

2. La durée de la fermentation 4 h. à 25°. L'osazone de la couche supérieure. P—3·96 %. N—20·54 %. L'osazone de la couche inférieure. P—4·21 %.

3. L'osazone de la couche supérieure 6 fois recristallisée. P—1·89 %. Lamelles jaunes. F. 141–142°. Il est à remarquer qu'après le traitement de la solution des éthers phosphorés avec de la phénylhydrazine à 100° on obtient souvent de très longues et larges lamelles jaunes qui après la recristallisation se transforment en aiguilles de couleur jaune ou orange. Il arrive cependant que la forme des lamelles persiste malgré plusieurs recristallisations, néanmoins le point de fusion ne change pas d'une manière sensible, en balançant dans des limites assez étroites autour de 142°, et la couleur reste toujours jaune foncée.

1. Supposant que les éthers phosphorés donnent des sels d'une différente solubilité, j'ai appliqué la précipitation fractionnée par l'acétate de plomb. Pour la première fraction j'ai pris à peu près une moitié de la quantité qui était nécessaire pour faire la précipitation complète des éthers. Le dépôt a été essoré et le filtrat traité de nouveau par l'acétate de plomb. Ainsi a-t-on obtenu une fraction qui traitée par de la phénylhydrazine a donné l'osazone de la composition suivante: N—15·96 et 16·28 (deux dosages); P—4·47; C—54·70 et H—5·63.

Il est curieux de noter ici que ces nombres, à l'exception de celui de C (l'osazone est très difficile à brûler), correspondent presque exactement à la formule:



$$\begin{array}{l} O\diagdown \quad \diagup O-C_4H_5(OH)_3-CN_2HC_6H_5-CHN_2HC_6H_5 \\ \qquad P \\ HO\diagup \quad \diagdown O-CH_2-CN_2HC_6H_5-CHN_2HC_6H_5 \end{array}$$

c'est à dire à l'osazone de l'éther phosphoré composé qui contient pour au moins[1] une molécule de H_3PO_4 une molécule de hexose et une molécule de triose.

	P	N	C	H
Calc. pour l'osaz. du hexose-triose-phosph.	4·51	16·28	57·56	5·38
Trouv. ,, ,, ,, ,,	4·47	16·12	54·70	5·63
Calc. ,, ,, hexose-biphosphate	5·68	15·38	52·75	5·68.

Je ne regarde point cette supposition comme démontrée, mais je ne doute pas que les recherches ultérieures fourniront un très important résultat.

Je donne ici 2 séries de nombres pour P et N des osazones obtenues dans les expériences, dont la température variait de 25° à 35° et la durée de 2 à $5\frac{1}{2}$ h. Après la fermentation pendant $9\frac{1}{2}$ h. on n'a pu obtenir *aucune* osazone. C'est la meilleure preuve que le mélange d'éthers se forme au début de la fermentation et qu'il est fermentescible; d'ailleurs on peut facilement le démontrer par l'expérience directe. Le maximum de la quantité d'éthers se forme à 35° après 2 h. et à 25° après 4–5 h., si l'on a, bien entendu, une levure très active.

Voici un exemple du rapport de la couche supérieure à la couche inférieure du liquide sirupeux. A 35° après 2 h. la couche supérieure contenait 15 cc., la couche inférieure—30 cc. Après 4 h. la couche supérieure—20 cc.; la couche inférieure—25 cc. La couche supérieure est plus soluble dans l'eau que la couche inférieure, de sorte que la quantité de celle-là dépend aussi de la quantité d'alcool employé pour la précipitation. D'autre part ces 2 couches se dissolvent en partie l'une dans l'autre, ce qui empêche extrêmement la séparation par des méthodes purement physiques.

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[1] Sous l'action de la phénylhydrazine une partie de H_3PO_4 pourrait se séparer de l'éther phosphoré

Dans de différentes expériences on a obtenu pour P et N des chiffres que l'on peut diviser en deux groupes.

1. 4·55, 4·37, 4·32, 4·29, 4·82, 4·85, 4·42, 4·21. Moyen: 4·48 % de P.
2. 5·48, 5·39, 5·84, 5·72, 6·01. Moyen: 5·69 % de P.

1. 21·20, 21·14, 19·12, 21·10, 20·54, 18·46. Moyen: 20·26 % de N.
2. 15·96, 16·65, 16·28, 16·82. Moyen: 16·43 % de N.

Le point de fusion variait entre 137° et 150° (toutes les osazones ont été recristallisées au moins une fois). Pour les osazones non recristallisées le point de fusion le plus bas a été 136°. Je rappellerai encore une fois que l'osazone du hexose-biphosphate a le point de fusion 150–152° et contient 5·68 % de P et 15·38 % de N. L'attention est frappée involontairement par le fait qu'on a obtenu dans quelques expériences des osazones qui contenaient 21·20 et 21·14 (deux dosages de la même substance) et 21·10 % de N. Ces nombres ne diffèrent que très peu de celui pour N de la triosazone, à savoir 20·9 %. Néanmoins, l'osazone qui a donné 21·20 et 21·14 de N contenait 5·84 % de P et avait le point de fusion 138–139° (recristallisée 5 fois); celle qui a donné 21·10 % de N contenait 5·31 % de P et avait comme point de fusion 141–142°. De sorte que si l'on se contente, comme on le fait souvent en analysant des osazones, de ne doser que l'azote, sans faire en outre attention au point de fusion, on peut croire qu'on aie à faire avec une triosazone.

Il est à remarquer que c'était justement moi [Lebedev, 1909, p. 123; 1910, p. 223; 1911, 1, p. 256] qui ai obtenu pour la première fois les hydrazones et osazones contenant du phosphore; avant cette découverte on pouvait bien facilement commettre une grave erreur.

Les chiffres pour P, 3·96, 2·96 et 1·89 %, disent en faveur de la supposition que le mélange d'osazones contienne une petite quantité d'osazone qui, ne contenant pas de P et étant moins soluble, s'accumule peu à peu à chaque recristallisation; cependant il est peu probable que ce soit l'osazone du triose, premièrement, à cause de sa grande solubilité dans l'alcool, deuxièmement, par le fait que les chiffres pour N, au lieu de devenir plus grandes, deviennent, au contraire, plus petites. On peut admettre que pendant l'action de la phénylhydrazine en solution acide à 100° une très faible partie du mélange des éthers se décompose en mettant en liberté le sucre qui donne ensuite l'osazone correspondante ou que l'acide phosphorique se sépare partialement du phosphate de l'osazone. J'observais depuis longtemps une chose pareille sous l'action de l'alcali faible sur l'osazone-phosphate; l'osazone qui s'en séparait avait le point de fusion 215° [Lebedev, 1910, pp. 218-9]. J'ai l'intention de faire plus tard des expériences analogues sur ce sujet.

Quoi qu'il en soit je n'ai jamais obtenu une osazone qui n'aurait pas contenu du phosphore; c'est pourquoi il ne peut y avoir aucune doute qu'Ivanov a fait une erreur [Ivanov, 1907] dans cette question, ce qu'il a d'ailleurs reconnu lui-même [Ivanov, 1915].

Il suit alors de tous les faits rapportés plus haut qu'en disposant les expériences d'une manière convenable on peut isoler un mélange d'éthers phosphorés dont les constituants varient probablement non seulement au point de vue quantitatif, mais aussi qualitatif, selon les conditions, comme, par exemple, la température, la durée de la fermentation, la quantité de sucre ou de phosphate, la réaction du milieu, etc.

Ivanov précipitait l'éther phosphoré après 24 h. par l'acétate de cuivre, mais après un laps de temps si long il pourrait arriver que l'éther avait été fermenté presque entièrement ou même totalement, de sorte qu'il n'y en restait que très peu ou même pas du tout dans le précipité. Ainsi dit-il que l'osazone obtenue après la fermentation du glucose avait au début un point de fusion 142°, mais après quelques recristallisations 127° seulement. Ce fait montre clairement qu'il y avait un mélange qui consistait probablement d'une très petite quantité d'osazone de hexose-biphosphate et d'une très grande quantité d'acétylphénylhydrazine qui se forme, comme on le sait, facilement sous l'action de la phénylhydrazine sur l'acide acétique. Les cristaux ne se purifient que difficilement et ont ordinairement une couleur un peu jaunâtre. Il est évident qu'Ivanov, faisant le dosage de N dans cette substance, a commis une erreur ayant obtenu 20·9 % au lieu de 18·7 %. Une telle erreur est possible si l'on n'est pas suffisamment habitué au dosage de N ou si la substance analysée n'était pas bien purifiée. Le mélange pourrait contenir, outre de l'hexose-phosphate, encore de l'hexose-triose-phosphate et triose-phosphate, sans parler d'autres éthers d'une pareille composition. Cette admission n'a aucun rapport au travail cité d'Ivanov, mais, comme j'ai déjà dit plus haut, a pour point de départ le fait, établi pour la première fois par moi, que la dioxyacétone se transforme pendant la fermentation en hexose-phosphate.

Aussi ai-je commencé des expériences dans lesquelles je me suis servi de la *p*-bromphénylhydrazine; bien que je n'aie fait que 2 expériences, il y a tout lieu à croire que la continuation de celles-ci aura une grande importance pour l'établissement de la formule des éthers, puisque on obtient les hydrazones en forme bien cristalline. La *p*-bromphénylhydrazone se forme en solution alcoolique après quelques minutes déjà; elle est de la couleur blanche de neige (F. 112–114°), mais après quelque temps, surtout sous l'action de la lumière, elle devient peu à peu jaunâtre, en se transformant évidemment en *p*-bromphénylosazone, ayant le point de fusion 141–142° (jaunes lamelles). Celle-ci, recristallisée une fois, donne de très minces et très longues aiguilles jaunes, entrelacées, qui au microscope ont un aspect de la ouate de verre. F. 143–145°. Je rappelle que les *p*-bromphénylhydrazone et *p*-bromphénylosazone, également obtenues pour la première fois par moi [Lebedev, 1909, 1910, 1911, 1], ont comme point de fusion 128° resp. 165° (non recristallisées). La hydrazone, étant presque insoluble dans l'eau, se dissout facilement dans $N/2$ NaOH avec une jolie couleur rosée. P—6·87 et 6·76 %; calculé pour *p*-bromphénylhydrazone du hexose-biphosphate—7·02 %.

Je n'ai pas encore analysé la *p*-bromphénylosazone. J'ai fait quelques analyses des sels amorphes de Ba, obtenus d'après 2 méthodes.

1. La solution du sel de Ba a été précipité par l'alcool à 97° en proportion 1 : 1; le précipité a été lavé par un mélange d'alcool et d'eau (1 : 1), dissous dans l'eau et précipité de nouveau par un volume égal d'alcool. Ba—41·55 %; P—8·30 %.

2. Le sel de Ba a été précipité par ébullition, filtré, lavé par l'eau bouillante, redissous et précipité de nouveau par ébullition. On a obtenu 40·87 % de Ba. Ba et P, calculés pour hexose-biphosphate, 44·92 % resp. 10·16 %.

Bien que les analyses de toutes les substances décrites plus haut ne soient pas encore complètes, on voit clairement qu'on a à faire avec un mélange au moins de deux éthers phosphorés. Par ce fait le cas devient difficile à résoudre, mais je ne doute pas que j'y réussirai tout de même, étant donné que toutes les substances, à l'exception des sels, sont cristallines et qu'on peut en avoir tant que l'on veut.

La réponse à la question de la décomposition du sucre dépend de la résolution du problème que je me suis posé, indépendamment des travaux de qui que ce soit, depuis si longtemps.

Je me réserve donc de continuer les recherches dans la direction indiquée dans ce mémoire.

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VIII. THE DIFFERENTIAL BEHAVIOUR OF THE ANTINEURITIC AND ANTISCORBUTIC FACTORS TOWARDS ADSORBENTS.

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Although we have as yet no definite information about the chemical identity of the antineuritic principle, several of its important properties have been disclosed by the researches of the last decade. Its behaviour towards certain adsorbents is an interesting feature. Chamberlain and Vedder [1911] observed that the antineuritic substance was removed by filtering through bone black. More recently Seidell [1916] has found that a pure preparation of fuller's earth, known as Lloyd's reagent, will also adsorb it and if used in suitable quantity will remove the active principle quantitatively, becoming at the same time activated itself. Williams and Seidell [1916] employed this procedure in order to isolate the active principle from autolysed yeast. They obtained a substance which was identical with adenine and which retained a certain degree of antineuritic activity for some time. Pure adenine from yeast has been shown however by Voegtlin and White [1916] and ourselves [1917] to be inactive. It is therefore probable that the adenine obtained by Williams and Seidell was only associated with the antineuritic factor in yeast in the same way as nicotinic acid has been shown to be associated with it in the extract of rice polishings [Barger and Ewins, quoted by Barger, 1914, p. 112; Drummond and Funk, 1914]. Both when purified become inactive. It is seen therefore that the adsorption procedure has not yet enabled us to isolate the looked for substance.

Very little is known about the chemical and physical nature of the antiscorbutic substance present in many plants and fruit juices, which is capable of preventing and curing scurvy. We know that it is not identical with the antineuritic factor, since substances showing marked antineuritic activity are antiscorbutically inactive and vice versa. There is also evidence that the antiscorbutic factor is more thermolabile than the antineuritic. It was our original intention to study the behaviour of the antiscorbutic towards adsorbents and in the event of adsorption to study its chemical nature. We have however found that unlike the antineuritic substance it showed under the conditions which have hitherto been employed no tendency to be adsorbed by the materials which removed the former. This differential behaviour of the two active principles towards adsorbents suggested to us the possibility of separating the two when present in the same solution. For want of a natural substance which would contain both in marked quantities we have mixed equal quantities of autolysed yeast and orange juice, the former being antineuritically very active, the latter rich in the antiscorbutic. We then found that we were able by means of fuller's earth quantitatively to remove the antineuritic substance, leaving the antiscorbutic unchanged. The description of the above experiments forms the object of this communication.

EXPERIMENTAL.

Mention was made above of Seidell's work on the adsorption of the antineuritic factor by fuller's earth and we have taken the opportunity of repeating the experiments. Being unable to procure Lloyd's reagent we made use of ordinary fuller's earth. Brewer's yeast after having the wort pressed out was autolysed at 37° and filtered through a Buchner funnel. The filtrate was then shaken for 15 minutes with fuller's earth (50 gms. of fuller's earth to 1 litre of the autolysed yeast), centrifuged, and the supernatant liquid decanted, this treatment being carried out three times. The resulting autolysed yeast fluid was tested curatively and protectively.

Exp. 1. The following experiment describes the action of the autolysed yeast after treatment with the adsorbent on a polyneuritic bird.

Pigeon E showed retracted neck and general paralysis. Received 5 cc. of the treated autolysed yeast. Next day very slight improvement noticeable. On the third day the bird had a relapse and a further 15 cc. was administered. As no improvement was noticeable during the day another 10 cc. was given at 3 P.M. on the same day. The bird was found dead next morning. All the doses were delivered direct into the crop by means of a stomach pump.

The above experiment shows that the autolysed yeast had been entirely inactivated by treatment with fuller's earth.

Exp. 2. For the protective experiment a batch of autolysed yeast was divided into two portions. One portion was treated with fuller's earth as described above while the other was left untreated. Four pigeons, *H*, *I*, *K*, and *L*, were used. All the birds were fed artificially with 80 gms. of rice a day in two portions of 40 gms. each. In addition the birds *H* and *I* received daily by means of the stomach pump 3 cc. of untreated autolysed yeast, while *K* and *L* were dosed with 3 cc. of the treated autolysed yeast. Fig. 1 gives a graphic representation of the changes of weight of the individual birds.

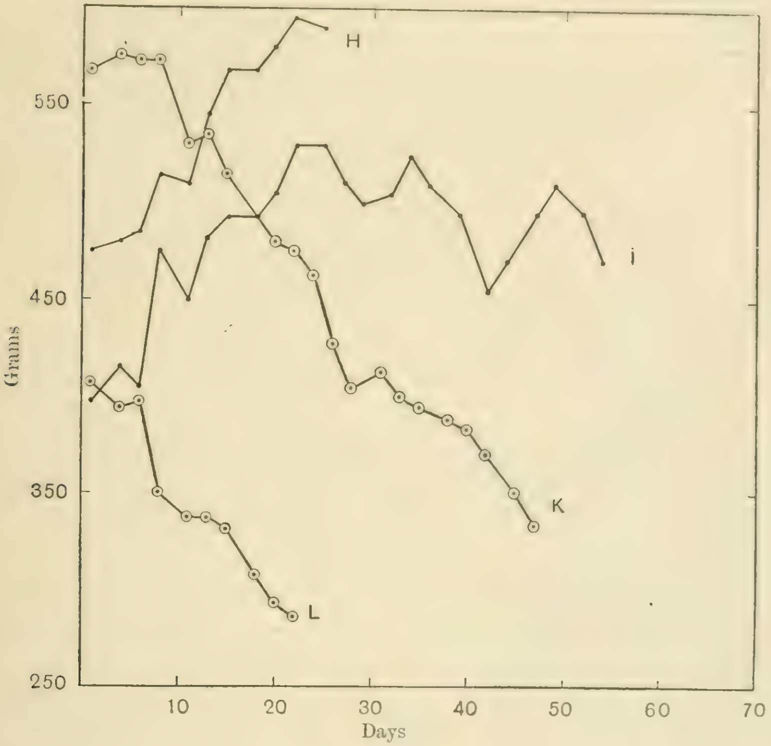

Fig. 1.

It is seen that while *H* and *I* did well on the treatment, even putting on weight, *K* and *L* gradually lost weight, the former succumbing after 46 and the latter after 21 days to polyneuritis. The bird *H* having injured itself had to be killed, while *I* was alive and well at the time the experiment was stopped.

The above experiments fully confirm Seidell's observations.

We have further observed that the active principle is also adsorbed when autolysed yeast is treated with dialysed iron.

Exp. 3. Our procedure was as follows. 200 cc. of autolysed yeast was shaken with 200 cc. of dialysed iron, and the precipitated iron compound was filtered and pressed with a hand-press almost to dryness. The mass was then shaken with a litre of 95 % alcohol for 3 hours, the alcoholic extract filtered and evaporated in vacuo at 45–50° and the residue taken up in 30 cc. of water. 10 cc. of the solution cured a polyneuritic pigeon. The following are the particulars of the case.

Pigeon 188 showed very grave symptoms of polyneuritis, convulsions and retracted neck. 10 cc. of the solution was given per os in the morning; gradual improvement during the day. Next day the bird was almost normal and received another 10 cc. of the extract. The following day the pigeon was quite normal and walked and flew freely. It was then put on a rice diet and developed another attack of polyneuritis seven days after the cure.

We have also observed that when autolysed yeast is treated as described above the filtrate still retains some activity. There is however little doubt that by treating the autolysed yeast with a suitable quantity of dialysed iron the entire content of the antineuritic principle could be removed as in the case of fuller's earth. We have not however as yet pursued the subject any further on these lines.

It has already been mentioned that our adsorption experiments with antiscorbutic solutions were primarily undertaken with the object of isolating or concentrating the active principle. The adsorbents that were able to remove the antineuritic factor from active solutions suggested themselves as suitable substances for the removal of the antiscorbutic. We therefore employed dialysed iron and fuller's earth for the purpose.

Exps. 4 *and* 5. 120 cc. of dialysed iron was added to 60 cc. of orange juice, which as is well known is a strong antiscorbutic, and shaken for some time. The precipitated iron was then filtered, pressed almost to dryness and shaken with 95 % alcohol for about 2 hours, the alcoholic extract being then filtered and concentrated in vacuo at a temperature of 30–40°. One third of the extract, which was equivalent to 20 cc. of the original orange juice, was used as a daily dose for an animal. The treatment with the fuller's earth was very similar. 10 g. of fuller's earth was employed for 60 cc. of orange juice, and after being shaken, filtered, and pressed out, was extracted with one litre of 95 % alcohol containing 0·5 % of citric acid for 2 hours. The alcoholic extract was concentrated in vacuo at 30–40° and, as in the case of the dialysed iron, one third of the quantity was used as a daily dose. Although 5 cc. of orange juice is known to be ample as a preventive dose in a guinea-pig we thought it advisable to employ the equivalent of 20 cc. in case the adsorption should turn out to have been incomplete.

We tested the antiscorbutic potency of our extracts on guinea-pigs. Holst and Frölich [1912] in their classical work have shown that if guinea-pigs are fed on certain diets they develop a condition which is pathologically identical with human scurvy. Oats, bran and water prove to be a very suitable diet for producing experimental scurvy in guinea-pigs and we have accordingly adopted it. In some of our later experiments, in view of the results obtained by Chick and Hume [1917], autoclaved milk was substituted for the water. We found that a daily ration of 50 cc. of autoclaved milk kept the pigs in a better condition but at the same time did not prevent the onset of scurvy. If guinea-pigs are fed on this diet they commence to drop in weight about 14 to 21 days from the commencement of the experiment and eventually die within a month. At the post mortem a pathological picture very much the same as that of human scurvy is disclosed. Subcutaneous and intramuscular haemorrhages, the latter specially pronounced in the thigh region, are well in evidence. Subperiosteal haemorrhages are also to be observed. The costochondral junctions in the ribs are abnormally enlarged

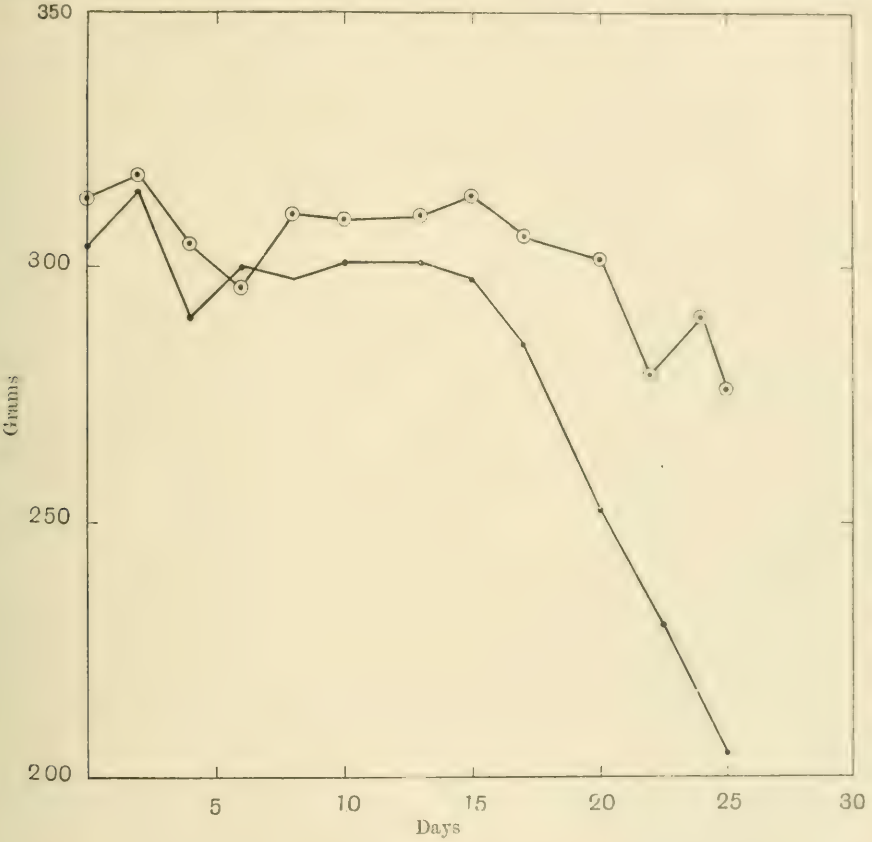

Fig. 2.

and haemorrhaged. The bones are very brittle and the teeth are loose. If the scorbutic diet is supplemented with an antiscorbutic, scurvy does not develop and the animal continues its growth in a normal way. For instance, a few cc. of orange juice given in addition to the oats, bran and autoclaved milk will keep a guinea-pig in excellent condition for months.

Our extracts were administered daily per os by means of a syringe, thus insuring quantitative consumption of the dose.

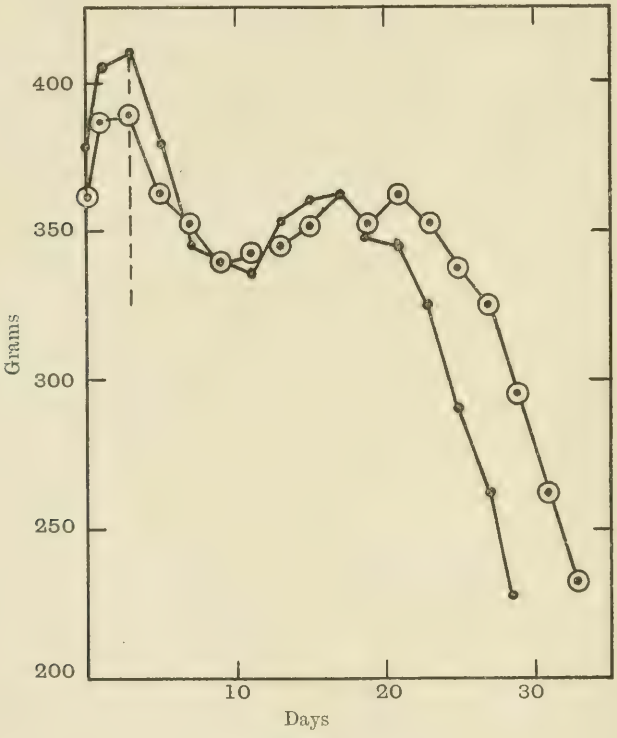

Fig. 3.

The vertical dotted line indicates the date at which the experimental treatment commenced.

Figs. 2 and 3 represent the experiments with dialysed iron and fuller's earth extracts of orange juice.

These curves show the weights of the guinea-pigs employed, two for each experiment. In both cases the animals dropped in weight as they do on a purely scorbutic diet and eventually died within the usual period. At the post mortem they all showed a condition of acute scurvy. The administered extracts neither prevented nor even delayed the onset of scurvy and we are bound to conclude that they were inactive.

The above experiments do not however entirely exclude the possibility of total or partial adsorption of the antiscorbutic by the adsorbents, as the solvents used, *i.e.* 95 % alcohol and acidified alcohol, may not have been suitable for extraction or the adsorption may have been of an irreversible character. To settle this point it was necessary to determine the activity of the solutions after being treated with the dialysed iron and fuller's earth.

For this purpose filtrates of orange juice treated with dialysed iron and fuller's earth respectively, according to the method already described, were tested. The method for the determination of the antiscorbutic potency of a substance is purely biological, and quantitative estimations cannot be carried out with the same degree of accuracy as are chemical estimations. There is very little doubt however that marked differences in the antiscorbutic content of solutions can be ascertained. For instance, we know that in the case of orange juice 3 cc. per day is about the minimum dose [Chick, Hume and Skelton, 1918] that will prevent the onset of scurvy in a guinea-pig and if an extract which is equivalent to 3 cc. of the original juice serves the same purpose we are safe in assuming that the loss of the antiscorbutic potency in the extract, if loss there be, is not very considerable.

Exps. 6 *and* 7. It was on these lines that we examined our filtrates. Figs. 4 and 5 show the weight curves of the animals employed.

Guinea-pigs 50 and 52 (Fig. 4) received orange juice treated with dialysed iron. Nos. 65 and 63 (Fig. 5) were dosed with orange juice extracted with fuller's earth, while No. 59 A (Fig. 5) served as a control for 65 and 63, receiving 3 cc. of untreated orange juice. None of the animals developed scurvy, showing that the solutions were active. It is further seen from Fig. 4 that guinea-pig No. 50, receiving an equivalent of 3 cc. of dialysed iron extract, did almost as well as No. 52, receiving 8 cc. As was previously mentioned, 3 cc. of ordinary orange juice is about the minimum preventive dose for a guinea-pig; consequently the antiscorbutic in the juice, if adsorbed at all, could not have been removed by the dialysed iron to any considerable extent.

The experiments with orange juice treated with fuller's earth gave similar results. No. 63, which received 3 cc. of the extracted juice, shows a weight curve almost identical with that of No. 59 A which received the same quantity of untreated orange juice. The juice in both cases emanated from the same batch. No. 65 received 8 cc. of the treated orange juice and developed somewhat better than the others. It must be mentioned here that all these animals were chloroformed at the close of the experiment and at the post mortem examination showed no signs of scurvy.

The above experiments show that the antineuritic and antiscorbutic factors behave differently towards the same adsorbents in their original media. It was of interest to see whether this differential behaviour held also true when the two principles were present in the same medium. We have so far not come across a natural product which contained both in quantities suitable for investigation and we therefore decided to make up a solution which would contain both in ample quantities and test it before and after adsorption.

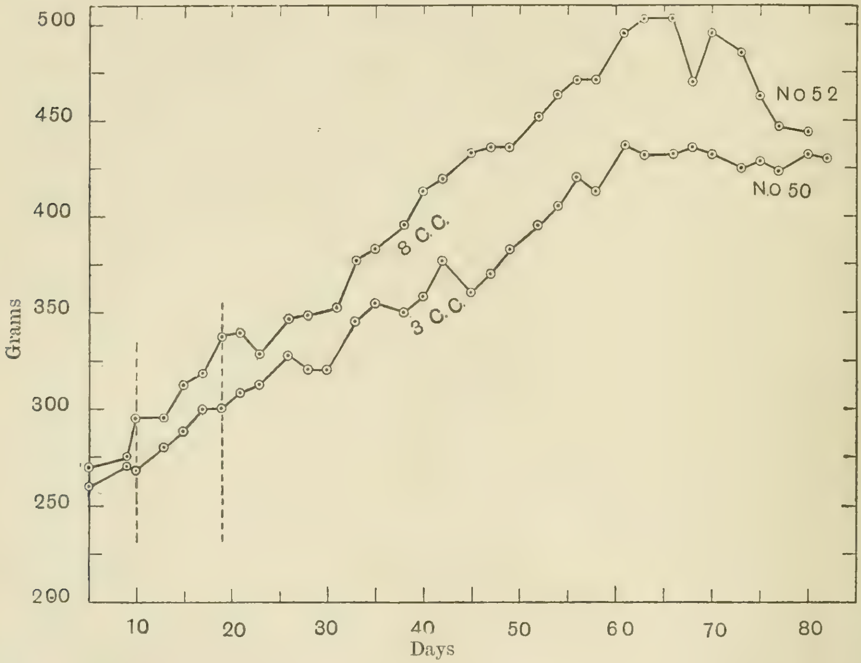

Fig. 4. The first vertical dotted line indicates the date on which the animals were put on a scorbutic diet; the second the date on which the experimental solutions were first given.

Exp. 8. A mixture of autolysed yeast and orange juice was therefore employed.

The yeast solution and the orange juice were mixed in equal quantities. One portion of the mixture was treated with fuller's earth three times; the other portion was employed untreated. Each portion was tested curatively and protectively for the antineuritic on pigeons and protectively for the antiscorbutic on guinea-pigs.

As would be expected, the untreated mixture proved effective in curing an acute case of polyneuritis; the mixture treated with the adsorbent however failed to show any antineuritic potency. The following protocol describes two cases of polyneuritic birds dosed with the treated mixture.

Pigeon C_2 showed paralysis and forward head. Received 10 cc. of treated mixture at 4.30 P.M. per os. Next day the bird was worse and another 10 cc. was administered. On the third day the bird was in a much worse condition and died at 2.30 P.M.

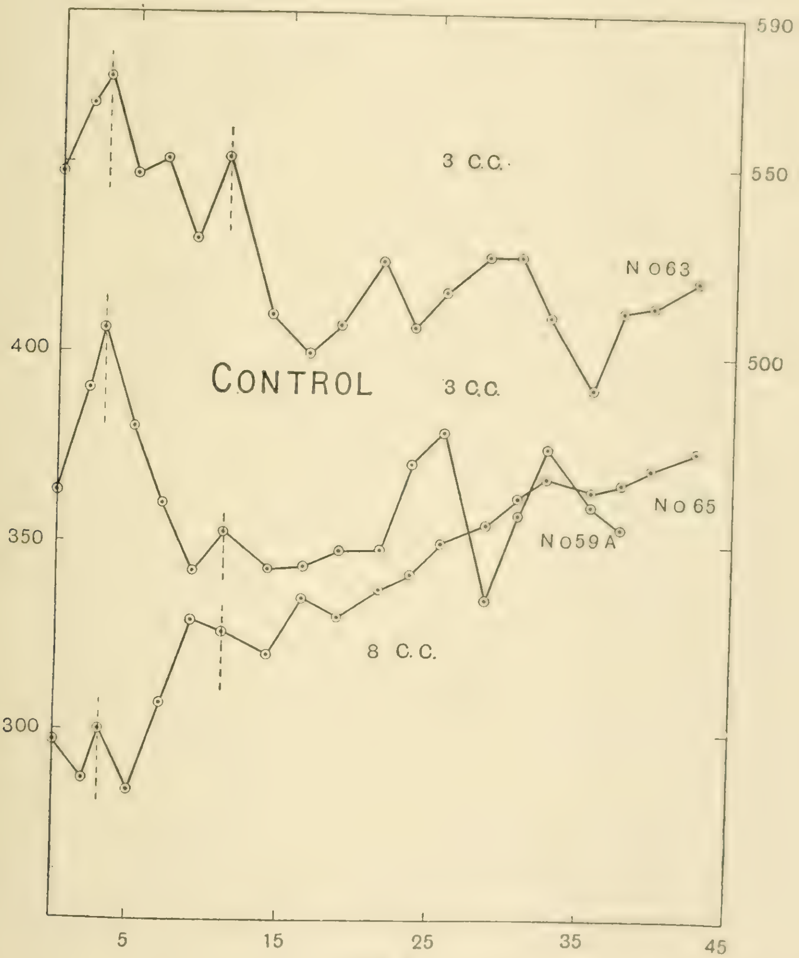

Fig. 5.

Pigeon D_2 was found in convulsions showing general paralysis with its head well forward. It received 10 cc. of the treated mixture at 10 A.M. No improvement during the day. Found dead the next morning.

For the protective experiment 10 cc. of the mixture, which was equivalent to 5 cc. of the original autolysed yeast, was administered daily to pigeons fed on an exclusive diet of polished rice. Four pigeons were employed. Pigeons A_2 and B_2 received the untreated, while pigeons A_1 and B_1 were dosed with the treated, mixture. A_1 and B_1 developed polyneuritis within 33 and 45 days respectively. A_2 and B_2 on the other hand showed no signs after 50 days. Fig. 6 represents the weight curves of these birds.

It will be seen that the birds in this experiment behaved very similarly to those in the experiment with autolysed yeast alone. Both from the curative and protective experiment it is evident that the treatment with the fuller's earth has inactivated the mixture as it did the unmixed autolysed yeast.

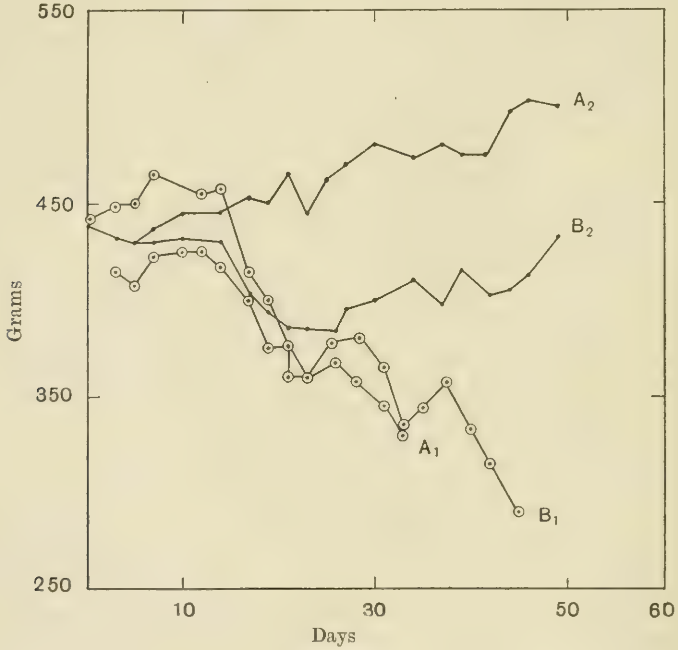

Fig. 6.

To test the antiscorbutic potency of the mixtures four guinea-pigs were employed. One received 6 cc. (equivalent to 3 cc. of orange juice) of the untreated mixture, the other three received 6 cc., 10 cc. and 12 cc. (equivalent to 3 cc., 5 cc., and 6 cc. of orange juice) respectively of the treated mixture. As will be seen from Fig. 7, all the animals developed normally. At the end of two months the guinea-pigs were chloroformed and at the post mortem they were all found in excellent condition without revealing any signs of scurvy. The treatment of the mixture then with fuller's earth has not affected its antiscorbutic potency to an appreciable extent.

We have performed one more set of experiments which had some bearing on the behaviour of the antiscorbutic principle towards adsorbents, namely the effect of filtration of an antiscorbutic through a porous Berkefeld filter. Expressed orange juice contains much suspended matter and is not a solution easily amenable to chemical manipulation. Filtration through filter paper is slow and the resulting filtrate is not very clear. When the juice however is passed through a Berkefeld filter by means of suction a brilliant, clear filtrate is obtained. It was of interest to ascertain whether any of the antiscorbutic had been adsorbed during the process of filtration.

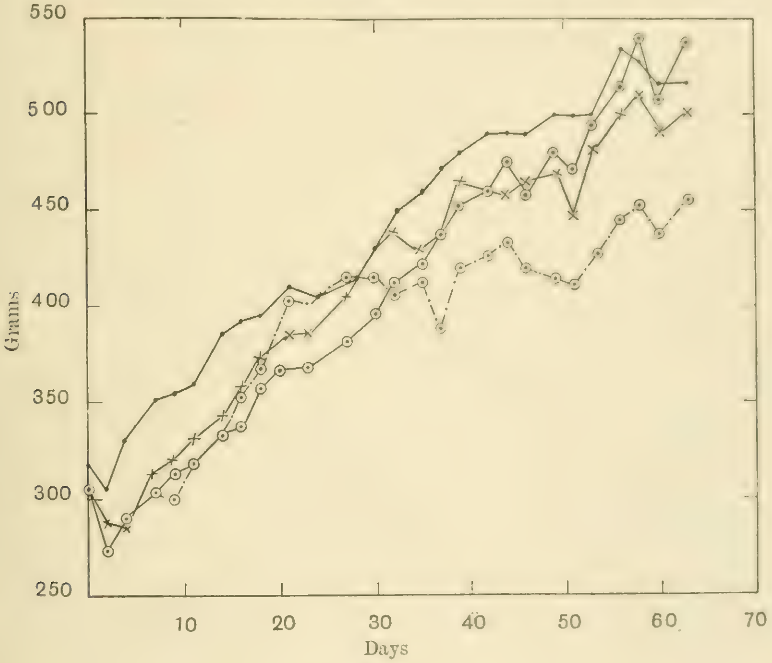

Fig. 7.

•——•——• 6 cc. treated mixture
⊙——⊙——⊙ 10 cc. ,, ,,
×——×——× 12 cc. ,, ,,
⊙—•—⊙—•—⊙ 6 cc. untreated mixture

Exp. 9. An experiment was instituted in which three sets of two guinea-pigs each received 3 cc., 5 cc., and 8 cc. of filtered and unfiltered juice respectively. Unfortunately the animal receiving the 3 cc. of the filtered juice succumbed to an unknown complaint 40 days after the experiment commenced. The post mortem did not reveal any signs of scurvy. The animals receiving 5 cc. and 8 cc. of filtered juice failed to develop scurvy and behaved very much in the same manner as the controls which were given the same quantities of the unfiltered juice. They were all chloroformed and no signs of scurvy were found at the post mortem. Fig. 8 gives a graphic representation of the experiment. These experiments show then that filtration through a porous medium like a Berkefeld filter does not affect the antiscorbutic potency of orange juice.

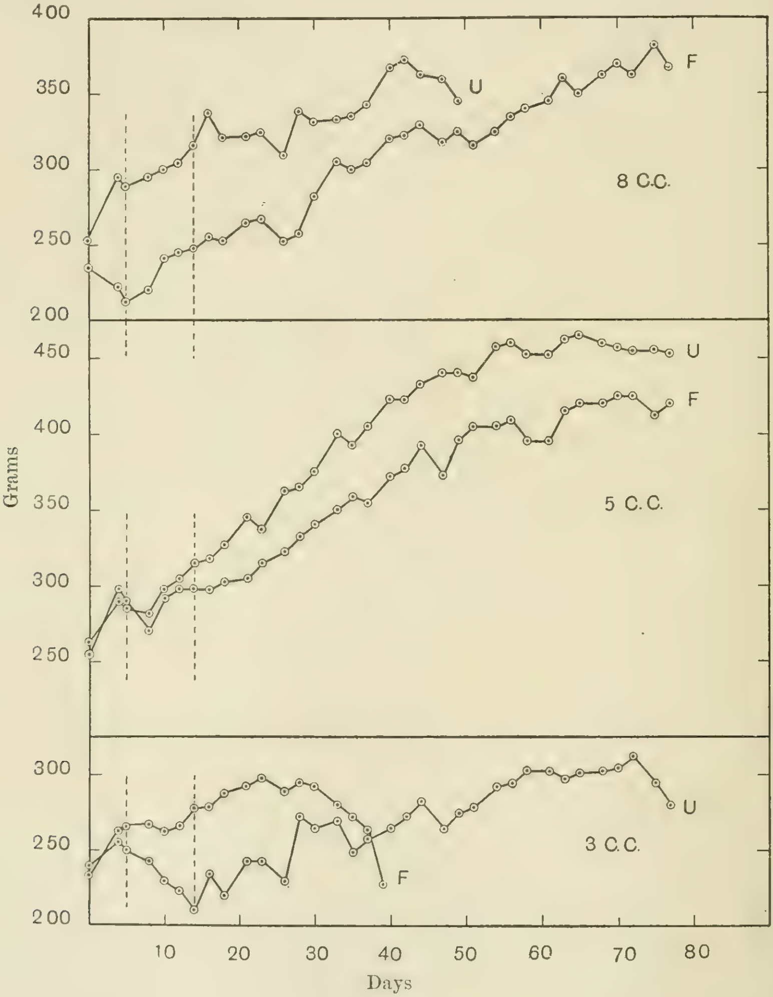

Fig. 8.

U, unfiltered F, filtered

CONCLUSIONS.

The experiments we have described show that under certain conditions at least the antineuritic factor behaves differently towards adsorbents from the antiscorbutic factor; the latter showing no tendency to be adsorbed by substances which remove the former from solution. We have shown that it is possible to separate the antineuritic principle from our artificial mixture containing both factors. Whether this is possible under all conditions we are not at present in a position to say. It is well known that adsorption is regulated by many factors and the H ion concentration of the medium is a condition to be considered. To work this problem out in detail is fraught with many technical difficulties. However it is quite within the limits of our present technique to investigate the behaviour of these accessory factors towards adsorbents in media differing in reaction in a marked degree and we are at present engaged in an investigation on these lines.

SUMMARY.

1. The authors have confirmed the fact that fuller's earth adsorbs almost quantitatively the antineuritic factor out of autolysed yeast.

2. Dialysed iron also adsorbs the antineuritic factor present in autolysed yeast.

3. Treatment with fuller's earth or dialysed iron does not affect appreciably the antiscorbutic potency of orange juice.

4. On treating a mixture of equal volumes of autolysed yeast and orange juice with fuller's earth the antineuritic factor is removed while the antiscorbutic activity remains unaltered.

5. Orange juice can be filtered through a Berkefeld candle without having its antiscorbutic activity vitiated to any appreciable extent.

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IX. ON THE NATURE OF THE PROTEOLYTIC ENZYME OF YEAST.

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The organism of the higher animals possesses a system of ferments hydrolysing the proteins to the stage of their final decomposition—to amino-acids. With regard to two components of this system—pepsin and erepsin [Cohnheim, 1901]—we have sufficient data to allow us to regard them as more simple ferments than animal trypsin. And indeed, pepsin converts proteins into peptones, erepsin acts on peptones, hydrolysing them into amino-acids, whilst trypsin unites in itself both these functions. The latter circumstance leads us to doubt the individual character of trypsin and allows us to regard it as a mixture of two ferments [Cohnheim, 1902; Salkowski, 1902]. However, trypsin cannot be regarded as a mixture of pepsin and erepsin, as, on the one hand, trypsin digests protein more slowly than pepsin, and on the other, it has been definitely shown that trypsin does not act on certain combinations of polypeptides, which are readily acted upon by erepsin.

In invertebrate animals, in which the differentiation of organs stands much lower, it is also much more difficult to observe the specific ferments. Here the hydrolysis of protein to amino-acids is attributed to tryptases [Oppenheimer, 1909, p. 222] which act most efficiently sometimes in an acid, and sometimes in an alkaline medium. Fredericq [1878], for instance, obtained from the tissue of a worm an enzyme which dissolved fibrin better in an acid medium (0·6–1·2 % HCl). We encounter phenomena of a similar kind in investigating the proteolytic ferments in plants. Owing to a weak physiological differentiation in plants it is difficult to solve the question, whether we are here dealing with one ferment hydrolysing the proteins to amino-acids, or whether there is also a system of ferments, which effects the hydrolysis of protein to the end by stages.

From the wide literature on this question I may mention the conclusion arrived at by Hahn [1903], who worked with the juice of yeast obtained by Buchner's method. Hahn thinks that the proteolytic ferment of yeast presents a new type of digestive ferments, as, similarly to pepsin, the best medium for its action is acid, whilst in its final products of decomposition it corresponds to the tryptic ferment. Hahn named the proteolytic ferment of yeast endotryptase since it acted inside the yeast cell, and although he was acquainted with the work of Cohnheim on ereptase, Hahn, and other investigators who preceded him, did not express themselves in favour of the complex character of the proteolytic ferments of plants. Butkewitsch [1904, see also Godlewski, 1911], who investigated autolysis of the seeds of *Lupinus angustifolius*, has shown that the process of decomposition of protein is inhibited in an acid medium (0·2 % HCl or 0·1–1 % HCN), and the correlation between the products of decomposition changes altogether, the greater part of them being precipitated by phosphotungstic acid. Evidently, the decomposition of proteins proceeds chiefly to peptones. The supposition that it is possible to alter the correlation between the products of decomposition of protein according to the conditions of the experiment, is corroborated by Fernbach and Hubert [1900]. These authors demonstrated that during the autodigestion of malt the products of decomposition were greatly influenced by the temperature. Thus, at 40° nearly the entire soluble nitrogen belonged to amino-acids, which are not precipitated by phosphotungstic acid. At 60° there were produced only 50–60 % of amino-acid nitrogen, and at 70° its amount did not exceed 40 %, the remaining quantity belonging to the peptones precipitated by phosphotungstic acid. Windisch and Schellhorn [1900] arrived at the same conclusion. The analogy involuntarily comes to mind that in the given case the phenomena taking place are similar to those in the fermentative metamorphosis of starch; and indeed, by heating diastase to 80° it is possible to destroy its function of sugar production, without influencing the dissolution of starch [Pottevin, 1899].

The quantitative alterations of the products of decomposition of protein, observed in different media and at different temperatures, indicate the possible presence in plant tryptase of at least two constituents. The numerous investigations of Vines ("The Proteases of Plants") on the proteolytic ferments of plants have provided experimental data on this question. Vines [1908] regards the protease of plants as an association of "peptase" and "ereptase." The first ferment dissolves the flakes of fibrin, and the second hydrolyses Witte peptone to products giving the tryptophan reaction. This author tried to obtain peptase and ereptase separately in extracts from leaves, bulbs and seeds. Thus, an extract from hemp seeds acidified with acetic acid produced a dense precipitate of protein, which on repeated dissolution in common salt

(NaCl) produced an opalescent solution, which was capable of digesting fibrin, but did not decompose Witte peptone to tryptophan; the filtrate from the protein precipitate contained an active peptonising enzyme (ereptase), but not "peptase." In the next paper [Vines, 1909] this author investigated papaïn in this respect. As regards the ferment of yeast, Vines [1902, 1904, 1905, 1906] discovered that a watery extract of dried yeast digested Witte peptone, but not fibrin, and an extract in 2 % NaCl digested both. The investigations of Vines are very illuminating, but his methods were far from perfect, and the author often sets forth unconvincing experiments for the confirmation of the correctness of his ideas. Thus, he founds all his views on the presence of ereptase in plants on the tryptophan reaction, whereas Butkewitsch [1904] had already shown that coloration by chlorine water does not present a criterion of the deep decomposition of protein, just as the reduction of an alkaline solution of copper oxide, due to the aldehyde group, does not correspond to the complete hydrolysis of the starch molecule to glucose, but appears in the dextrins together with the liberation of their aldehyde group. How careful one has to be in applying the tryptophan reaction for the establishment of the deep decomposition of protein is shown in the experiments of Levene and Rouiller [1907]. These authors decomposed the protein by barium oxide solution and observed coloration by bromine water even after slight hydrolysis; but in the latter case *the substance producing the tryptophan reaction was precipitated wholly by phosphotungstic acid.* As it was impossible to separate tryptophan from such a precipitate, the authors arrived at the conclusion that tryptophan was here in the form of a constituent of a complex compound, probably a polypeptide. Since the polypeptides manifest the tryptophan reaction, it cannot be used for the detection of ereptase at all. Of course, it was also impossible to identify the ereptase with Cohnheim's erepsin, as for this purpose it would have been necessary to show that ereptase is capable of hydrolysing combinations of amino-acids which are inaccessible to trypsin. In general, for the sake of uniformity, Vines' terms ought to be retained and a rational nomenclature of ferments adopted. The ferment splitting the peptones to amino-acids should be named, in agreement with Oppenheimer [1909] *peptase*, without deciding *à priori*, whether it is to be identified with Cohnheim's erepsin, or with one of the constituents of animal trypsin acting on peptones. The ferment which dissolves the protein and converts it into peptone should be named *protease*, using this term in a restricted sense, and not denoting by it the sum of proteolytic ferments. Thus, owing to Vines, we are able to distinguish in plant trypsin at least two constituents, the protease and the peptase. As regards the protease, which is equivalent in its significance to pepsin, it became known after the fundamental investigations of Hooker [1874] and Darwin [1888] on insectivorous plants. These authors arrived at the conclusion that the secretions of insectivorous plants contain an enzyme similar to the *pepsin* of animals, *i.e.* digesting fibrin and other proteins in an acid medium. After the detailed investigations of Vines [1897, 1898, 1901] on the juice of *Nepenthes*, the experiments of Abderhalden and Terruchi [1906] have shown that this juice, just as in *Drosera*, does not hydrolyse the peptides, *i.e.* is identical with the pepsin of animals. Thus, the presence of pepsin in insectivorous animals points to a high degree of physiological differentiation, since pepsin appears only in the higher animals, whilst in the invertebrate animals it is either not observed at all, or its presence is disputable. The idea of the identity of pepsin in plants and animals (the "pepsin-idea" of Vines [1909]) was the starting-point in the investigation of the proteolytic ferments of plants, and only after Green [1890] had discovered in seeds a ferment hydrolysing proteins to amino-acids could be added to it the "trypsin-idea." At present Abderhalden's method [Abderhalden and Schittenhelm, 1906; Abderhalden and Dammhahn, 1908; Abderhalden and Rilliet, 1908; see also Euler, 1907], applied by himself and his pupils, has assumed great importance in the study of peptase in plants. It is founded on the change in the rotation of the solution of optically active polypeptides after the addition of the ferment. By this method it was possible to demonstrate [Abderhalden and Brahm, 1908] that, if the juice of yeast is diluted in a certain manner, it hydrolyses added polypeptides, similarly to intestinal juice, quite identically and at the same rate; the peptase of yeast could thus be identified with Cohnheim's erepsin.

In my experiments on the proteolytic ferments of yeast I adopted Stutzer's method of estimation of nitrogen in protein. This method of quantitative estimation of nitrogen in the precipitate of copper hydroxide has evoked wide criticism. The most essential arguments were raised by Schjerning [1900], who directed attention to the facts that peptones were not precipitated by copper, and that amides passed into the precipitate. However, in my experiments amides are not encountered in the process of autolysis of yeast and the peptones in the filtrate may be determined by other methods. Stutzer's method is inapplicable only in the presence of a large quantity of carbohydrates, for recently Kostytschev and Brilliant [1916, see also Maillard, 1913] have pointed to a remarkable fact—the union of amino-acids with sugar without any intervention of ferments. This product gave with copper hydroxide a compound insoluble in water. In other cases Stutzer's method gives good results; it should only be remembered that *from the diminution of nitrogen in the precipitate of copper hydroxide the conversion of proteins can be guaranteed only to peptones*, as they are no longer precipitated by copper. For the estimation of the work of peptase, *i.e.* the conversion of peptones into amino-acids, other methods are required, and I estimated the nitrogen in amino-acids by means of the improved apparatus of van Slyke [Plimmer, 1908]. By combining both these methods it was possible to determine the action of both protease and peptase. My experiments were conducted either with hefanol (a Munich preparation of dried yeast), or with dried yeast (Lebedev). In 1912 I published the results of my experiments on the influence of phosphates upon the proteolytic decomposition during autolysis of yeast. Using different temperatures, adding K_2HPO_4 or KH_2PO_4 for the production of alkaline and acid media, I estimated in the filtrate—after treatment by copper, according to Stutzer—the amount of nitrogen in the precipitate produced either by lead acetate or phosphotungstic acid. It was found that the acid phosphate (KH_2PO_4) had the effect of considerably increasing the decomposition of protein, judging by the precipitate of copper oxide; however, additional precipitation of the filtrates by phosphotungstic acid showed that in proportion to the surplus amount of protein decomposed in the phosphate portion, the surplus of nitrogen in the phosphotungstic precipitate also rose. Thus after 67 hours' autolysis of hefanol at 52–58° the amount of nitrogen in the *normal portion* was:

in the copper precipitate	145·7 mg.
in the phosphotungstic acid precipitate	131·1 ,,
Total ...	276·8 ,,

and in autolysis with 1 % KH_2PO_4:

in the copper precipitate	118·9 mg.
in the phosphotungstic acid precipitate	163·2 ,,
Total ...	282·1 ,,

The same results were obtained when lead acetate was employed for precipitation, instead of phosphotungstic acid. Assuming with Vines the presence of two constituents in the proteolytic ferment of yeast, I had already at that time arrived at the conclusion that *KH_2PO_4 increases the decomposition of protein only to the stage of the high-molecular products of decomposition of protein—peptones*—which, as is known, are precipitate dboth by phosphotungstic acid and lead acetate. Therefore, in the case quoted we can speak only of stimulation of protease[1].

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[1] In my work of 1912 I used Vines' terms "peptase" and "ereptase," which must now no longer be employed.

In order to ascertain this point finally I have estimated the quantity of amino-groups by van Slyke's method in the normal and phosphate portions.

Exp. 1. Four portions of hefanol of 1·1 g. were set for autolysis with 25 cc. of water at 46°. To 2 and 4 were added 0·37 g. KH_2PO_4 in each and to all toluene. The experiment was conducted for 43 hours. The initial quantity of protein-nitrogen in the portions varied between 83·4 mg. and 84·2 mg.; the amounts of nitrogen obtained are, for the sake of convenience, re-calculated to portions containing 100 mg. of protein-nitrogen. In 1–2 the nitrogen was estimated according to Stutzer, in 3–4 according to van Slyke.

Nos. of portion	Composition of portion	Decomposed protein in mg. of protein-nitrogen	Newly formed amino-acid nitrogen
1 and 3	Water	37·8	14·6
2 and 4	1·5 % KH_2PO_4	51·8	14·9

Here, notwithstanding the greatly increased amount of protein decomposed in the presence of acid phosphate, the quantity of nitrogen according to van Slyke, *i.e.* the nitrogen of amino-acids, did not increase. It appears that *the given conditions have created surroundings favourable for protease, but these conditions (acid medium) have in no way accelerated the work of peptase.*

Saleski and Schatalov [1915], who have revised my previous experiments with phosphates, have arrived at the same results, but they could not explain the fact that in my experiments the difference between the normal and phosphate portions did not disappear with time, and at the end of autolysis remained the same as after 21 hours. Indeed, after 46 hours of autolysis at 54–58°, the decomposition of protein in my experiments was in the normal—58·5 %; phosphate—73·5 %, and after 72 hours—normal—56·9 %; phosphate—73·1 %. In all cases, when autolysis was conducted at a higher temperature, rising to 58°, this difference was maintained, but at a temperature of 34° the difference between the normal and phosphate portions disappeared after 7 days, and at 18° autolysis during 10 days gave:

normal—69·6 % of protein-nitrogen,
phosphate—68·4 % of protein-nitrogen.

This phenomenon is explained by the fact that *at high temperature the work of peptase is at first interrupted, and later altogether suppressed.* The high-molecular product of protein decomposition accumulated in these conditions is not hydrolysed further; that is why this difference is maintained.

Exp. 2. Portions of about 1 g. dried yeast (Lebedev) with 10 cc. water were set for autolysis with toluene. Amount of protein-nitrogen—100 mg. Temperatures—34° and 54°.

Nos.	Conditions of experiment	T	Amount of amino-acid nitrogen in mg. in portion	 newly formed
1	Autolysis 90 hours	34°	54·9[1]	—
2	Same, added 0·2 cc. 10 % NaOH and 20 hours longer	34°	55·0	—
3	90 hours, added 0·25 g. peptone and 20 hours longer	34°	63·2	8·3
4	90 hours, added 0·25 g. peptone + 0·2 cc. 10 % NaOH, and 20 hours longer	34°	66·9	12·0
5	Same as 1, but higher temperature	54°	39·8[1]	—
6	Same as 3, but higher temperature	54°	41·2	1·4
7	Same as 4, but higher temperature	54°	42·2	2·4

[1] To each of the control portions 1 and 5 was added 0·25 g. peptone previously to nitrogen estimation by van Slyke's method.

It follows clearly from the experiment that during 90 hours at 34° the peptase is not destroyed, as the peptone added is hydrolysed by it; this is seen from portions 3 and 4, in which an increase in the nitrogen, according to van Slyke, is observed, especially in the presence of a small amount of alkali. Autolysis at 54° has interrupted the work of peptase, and later has altogether inactivated it, as the peptone added was hardly decomposed, and there was no increase of amino-acid nitrogen, disregarding the 1·4 and 2·4 mg., which are due to a certain decomposition of peptone in water solution at the temperature of 54°, as control experiments have shown.

That acids and acid salts promote the work of the proteolytic ferment has been known since the time of the first investigations of Gorup-Besanez, but from the experiments with acid phosphate we observe that only the work of protease is increased, *i.e.* the conversion of proteins to the high-molecular products of decomposition. In animal physiology it is assumed that under the influence of hydrochloric acid the proteins are converted into acid albumin soluble in water and in this form become easily acted upon by pepsin. The data of Neumeister [1890] prove that before being digested by pepsin the coagulated protein is simply dissolved without any traces of denaturation. The addition of KH_2PO_4 in my previous experiments also evoked the dissolution of coagulated protein in the presence of the ferment. Thus, a watery extract of hefanol containing a large amount of heat-coagulable protein was submitted to autolysis with and without KH_2PO_4, at temperatures of 32° and 54°. At 32° the experimental portions were quite transparent and the difference in the decomposition of protein was insignificant (54·3 % control and 64·5 % in phosphate). At 54° the control produced 38·5 % decomposition and *contained a precipitate of coagulated protein*, whilst the phosphate was transparent and the decomposition of protein in it was 65·1 %. The presence of

KH_2PO_4, probably, evoked the formation of acid albumin and thus accelerated the work of protease; the importance of the acid phosphate was considerable at 54°, when coagulation of the protein took place. Besides this rôle, KH_2PO_4 could also serve to convert the pro-enzyme stage of protease into the active form, similarly to the manner in which the pro-pepsin (pepsinogen) of animals is transformed to pepsin by acids. My previous experiments have proved with full evidence that during autolysis at temperatures of 19° and 54° the final products of decomposition of protein will be different. At these lower temperatures hardly any intermediate high-molecular products of decomposition of protein[1] are observed: if the filtrate from the copper treatment is precipitated with lead acetate. the precipitates obtained in the portion autolysed at 19° only one-fifth the size of those obtained at 54°. That shows that at a high temperature the work of peptase is interrupted: this is corroborated by *Experiment* 3. After standing with toluene at 34° peptone does not increase the amino-acid nitrogen but at 54° this is observed to a small degree.

Exp. 3. Two portions of dried yeast (Lebedev) to the amount of 1 g. were set for autolysis with 25 cc. water. Toluene added; duration 94 hours. Temperatures 34° and 58°.

T.	Protein decomposed in mg.	Newly formed amino-acid nitrogen
34°	68·8	42·6
58°	50·4	20·9

Therefore, *to each* 100 *mg. of protein decomposed at* 34° *correspond* 61·8 *mg. amino-acid nitrogen*, and to the same amount decomposed *at* 58° *there correspond only* 41·5 *mg.* Since it is possible to estimate the action of peptase by the amount of amino-acid nitrogen, it is clear that high temperature inhibits its work.

In my preceding work I reduced the proteolytic ferment of yeast to an inactive state by heating to 80° in water. Hefanol heated in this manner did not produce decomposition of protein during further autolysis, but if acid phosphate (1·5 %) was added to the experimental portion. the decomposition of protein was again observed. In one experiment out of 106·4 mg. of protein-nitrogen, 5·9 mg. were decomposed; in another, in which the temperature was raised to 75°—even 21·3 mg. This decomposition may be ascribed either to the regeneration of the ferment caused by the acid salt, or, which I think more probable, to the conversion of the pro-enzyme under these conditions into the active form. The pro-enzymes are in general more resistant to heating than the ferments themselves. In order to demonstrate that the decomposition of protein takes place here owing to the action of the ferment, and is not due to the influence of the acid phosphate, I carried out experiments with boiled portions and portions inhibited with formalin.

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[1] This is also mentioned by Hahn [1903], who found on autolysis traces of albumoses and a total absence of peptones.

Exp. 4. Portions of hefanol of 1·1 g. set for autolysis at 46°, toluene added; 1–2 were treated with 25 cc. boiling water and boiled during 1 minute; to 3–4 were added 25 cc. 1·5 % formalin. Into each of the portions 2 (after cooling) and 4 was introduced 0·37 g. KH_2PO_4. Autolysis during 42 hours.

Nos. of portions	Condition of experiment	Amount of protein-nitrogen *previously to experiment*	Amount of protein-nitrogen *after experiment*
1	Boiled	84·2	84·4
2	Boiled + KH_2PO_4	83·8	83·9
3	1·5 % formalin	84·9	84·1
4	1·5 % formalin + KH_2PO_4 ...	84·4	84·5

In the result no traces of decomposition of protein were observed even in the phosphate portions after the ferment was inactivated either by boiling or by formalin. What were the products of decomposition of protein obtained after the resumption of the activity of the proteolytic enzyme under the influence of KH_2PO_4 after heating at 75–80°? The question was, whether there took place a transition from the pro-enzyme state of protease and peptase, or only from the former. In my preceding work it was observed that in proportion as the decomposition of protein with phosphate was increased, the greater was the amount of nitrogen appearing in the precipitate with lead acetate, as compared with the control portion. I have arrived at the conclusion that in the given case one may only speak of the work of protease carrying out the decomposition of protein to the high-molecular products of digestion. That heating to 75° destroys peptase completely is seen from the following experiment.

Exp. 5. Portions of dried yeast (Lebedev) of 1 g. were heated 1·75 minutes to 75° on a water-bath with 25 cc. water. To each of 2 and 3 was added 0·25 g. peptone.

1. Control, nitrogen estimated according to van Slyke ... ... 13·0 mg.
2. Same, but previously to estimation 0·25 g. peptone added ... 15·7 ,,
3. To portion heated to 75° added 0·25 g. peptone, and left for 20 hours at 56°. ... ... 16·0 ,,

Notwithstanding the 20 hours' digestion at 56°, the amount of amino-acid nitrogen did not increase, as compared with control portion 2. Therefore, heating to 75° destroys peptase, and only the protease is regenerated in the given conditions under the influence of KH_2PO_4.

This is seen from the following.

Exp. 6. Four portions of hefanol of 1·1 g. each with 25 cc. water were previously heated on the water-bath for 1·5 min. to 75°; then after cooling, to each of 2 and 4 were added 0·37 g. KH_2PO_4 and these allowed to remain 44 hours at 46°. In all toluene. Amount of protein nitrogen estimated to 100.

Nos. of portions	Nature of portion	Nitrogen in precipitate of $Cu(OH)_2$	Protein decomposed, in mg. of N	Newly formed amino-acid nitrogen
1 and 3	Control	100	0	0
2 and 4	Phosphate	89·6	10·4	0·8

Only the work of protease is present; decomposition was conducted only to the high-molecular products not precipitated by $Cu(OH)_2$, but precipitated by lead acetate.

Exp. 7. Same as preceding, but the portions were precipitated with lead acetate after autolysis.

Nature of portion	N in mg. before experiment	Nitrogen in lead acetate precipitate
Control	70·1	69·7
Phosphate	72·0	71·9

No difference was obtained between the control and phosphate portions, as the high-molecular products of decomposition, protein-albumoses and peptones, produced in the latter case, were precipitated by lead acetate.

In the experiments described above the same phenomenon was repeated as in the autolysis of yeast, in which the ferment was not inactivated by heating; in both cases in the phosphate portions was observed an accumulation of surplus high-molecular products of decomposition, but the quantity of amino-acids did not increase.

Involuntarily there come to mind the analogous results with the fermentative conversion of starch into glucose. I have already mentioned experiments in which by heating to 80° it was possible to inactivate the sugar-forming function of diastase without hindering the solution of starch and its conversion into dextrins. Evidently, in my experiments a complete inactivation of peptase also takes place. My data can be placed in parallel with the experiments on papaïn. Papaïn is regarded as a ferment of the tryptic type and Vines even discovered in it both constituents of the proteolytic enzyme. However, a series of authors [Delezenne, 1906; Jonescu, 1906; Sachs, 1907] had shown that papaïn digested egg-white best at 80–90°; *decomposition proceeds rapidly, but is carried on only to albumoses and peptones.*

While I dealt only with killed yeast—hefanol and zymin—I invariably obtained activation of the protease of yeast by acid phosphate, only sometimes in portions to which phosphate had not been added there was also observed a slight decomposition, probably due to incomplete inactivation of the ferment by heating. But when I carried out similar experiments with dried yeast (Lebedev), it occurred that *rapid* heating to 80° inactivated the ferment altogether, and after gradual heating during 2 min. to 75° decomposition of protein was observed both in the phosphate and control portions, but there was produced no increase of the amino-nitrogen in this decomposition.

Exp. 8. Portions of dried yeast (Lebedev) of about 1 g. with 25 cc. water, heated for 2 min. to 75°[1]. Autolysis with toluene 20 hours at 45°. To each of 3 and 4 was added 0·37 g. KH_2PO_4 after cooling.

Nos. of portions	Nature of portion	Decomposition of protein in mg. of nitrogen	Newly formed amino-acid nitrogen
1 and 2	Watery	14·0	0
3 and 4	Phosphate	16·6	0

It is seen that the addition of phosphate hardly produced any effect; this is a general phenomenon in working with dried yeast (Lebedev), as even during autolysis of this yeast with the ferment *not inactivated by heating*, no increase of the decomposition of protein in the presence of KH_2PO_4 was observed. Probably, in the given case, the yeast possessed highly active enzymes, and, therefore, the addition of KH_2PO_4, which in its essence serves as an activator, does not alter its work. It must also be noted that in Experiment 8 the product of decomposition of protein is nearly wholly precipitated by phosphotungstic acid; thus the amount of protein decomposed, expressed in mg. of protein-nitrogen, was 14·0, and in the phosphotungstic precipitate following copper, 11·9 mg. of nitrogen. This fact confirms the assumption that here are produced the primary products of decomposition of protein, the so-called albumoses and peptones. These primary products of decomposition are obtained in large quantities during autolysis of short duration; thus, during 3 hours' autolysis of dried yeast (Lebedev) at 45° in my experiment, 32·1 mg. of protein were decomposed, and only 2·1 mg. of amino-acid nitrogen were newly formed; or to 100 mg. correspond only 6·2 mg.

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[1] It was proved by control experiments that during the 2 min. heating no visible decomposition of protein took place.

Besides high temperature, with the view of depressing the work of peptase I have conducted experiments on autolysis of dried yeast (Lebedev) in the presence of alcohol.

Exp. 9. Portions of dried yeast (Lebedev) of about 1 g. with 20 cc. water + 5 cc. 95 % alcohol. Autolysis during 20 hours at 45°.

Amount of protein decomposed in mg. of N	18·2.
Newly formed amino-acid N	0.

Thus, 19 % alcohol destroys the work of peptase similarly to heating.

In all my experiments which were carried out either in a natural slightly acid medium, or acidified, owing to the presence of KH_2PO_4, I have not observed any stimulation of peptase; on the contrary, after a certain time, especially at high temperature, it was altogether inactivated. In the work of 1914 [Ivanov, 1914] I changed the acid medium of the experimental portion to alkaline during the autolysis of yeast, and allowed the decomposition of protein to proceed. I expected that in the alkaline medium, which is most favourable to animal trypsin and peptase [see experiments of Abderhalden and Terruchi, 1906], the decomposition of the intermediate compounds to amino-acids would proceed better; as a matter of fact I observed, on such a change of medium, accumulation of nitrogen determinable by Stutzer's method, and a corresponding diminution of nitrogen in the lead acetate precipitate, in which the presence of peptones could have been suspected. I at that time arrived at the conclusion that in the given conditions there was a possibility of fermentative synthesis from the products of primary decomposition of proteins. However, the question proved to be more complicated: if the medium became weakly alkaline from a slight addition of secondary phosphate (K_2HPO_4), then, as I have previously shown [Ivanov, 1916], in an alkaline medium at the cessation of the decomposition of protein increased formation of amino-nitrogen was observed, *i.e.* the work of peptase. Thus, in one experiment after complete cessation of the decomposition of protein, the amount of amino-acid nitrogen rose from 78·8 mg. to 102·2 mg. after an addition of alkali and renewed autolysis during 24 hours. At that time I concluded that toward the end of autolysis "there is accumulated a certain amount of polypeptides not precipitated by copper, and with difficulty acted upon by ferments; the addition of alkali and high temperature evoke a retardation in the decomposition of protein, but facilitate the decomposition of these polypeptides."

Thus, it was possible to separate the two constituents of the proteolytic ferment by function; *in this case, in the alkaline medium the protease was inactive, while the peptase acted*; and in the experiments described above the peptase was inactivated by heating to 75° and only the protease manifested activity in the presence of acid phosphate.

In the above-mentioned work of 1916 I succeeded in establishing the presence of phenylalanine amongst the products of decomposition of protein in alkaline medium. E. Fischer and Abderhalden [1903] consider that during digestion of protein by animal trypsin, phenylalanine (and proline) never appear amongst the products of decomposition, on account of the polypeptides which contain them being resistant to the action of this ferment. And since the hydrolysis of these polypeptides is possible only by erepsin, the peptase of yeast may be regarded as identical with the erepsin of animals.

After the conclusion of the present investigation, I received the recently issued excellent paper by K. Dernby [1917]. The author applied exact methods for the determination of the concentration of the H-ions of the medium and demonstrated that there exist corresponding optima of the concentration of H-ions for pepsin, trypsin and ereptase of yeast. Thus, according to Dernby, the proteolytic ferment of yeast does not consist of two constituents —protease ("pepsin") and ereptase, as might be supposed, on account of previous experiments, but of three; to them is added also tryptase, which is analogous in action to the trypsin of animals.

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X. THE ACETONE BODIES OF THE BLOOD IN DIABETES.

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The analyses of blood given below were made some years ago, but were not published as the method of estimation of aceto-acetic acid employed was found to be unsatisfactory. When known amounts of sodium aceto-acetate are added to urine, a yield of from 97 to 100 % of the theoretical amount of acetone can be obtained by the method published previously [Kennaway, 1914, 1]. But when the same method is applied to blood, a yield of no more than 88 % is obtained, and no means have been found of overcoming this defect. β-Hydroxybutyric acid shows no such difference in behaviour in blood and in urine; possibly the greater chemical activity of aceto-acetic acid, which is indicated by its constitution [Hurtley and Trevan, 1916], causes it to combine with some constituent of the blood which is not present in urine.

However, no results have been found in the literature which have been proved to be more accurate than those here brought forward; the analytical methods employed previously have been tested not with aceto-acetic acid, but with acetone, and the recovery of acetone from blood is a comparatively easy matter. Moreover, in a recent paper Hurtley [1916] has drawn attention to the great lack of any information on the amounts of acetone bodies present in the blood in diabetes; the analyses of Geelmuyden and of Marriot appear to be the only ones to be found in the literature. Geelmuyden [1909] estimated the acetone bodies in the blood obtained post-mortem from two cases of diabetic coma; in his earlier series [1904] the β-hydroxybutyric acid was not estimated. Sassa [1914] estimated the β-hydroxybutyric acid alone in the organs of three diabetics, but did not examine the blood. Marriot [1914] gives the amounts of acetone bodies obtained by his nephelometric method in the blood, withdrawn during life, from eight cases of diabetes; only one of these showed any signs of impending coma. The results from this case, together with those of Geelmuyden, are appended to Table II below for comparison.

METHOD OF ESTIMATION.

The estimations were made by the combination of the Schaffer and Scott-Wilson method described previously [Kennaway, 1914, 1] for the estimation of acetone bodies in urine. As a rule the addition of basic lead acetate solution (U.S.P.) and ammonia (sp. gr. 0·88) in the proportion of 60 cc. of the former and 25 cc. of the latter for 50 cc. blood produces a perfectly clear colourless filtrate; if too little ammonia be added the filtrate is coloured with reduced alkaline haematin and froths on distillation. The first distillates are redistilled with caustic soda and hydrogen peroxide as described by Schaffer [1908]. This method gives satisfactory agreement between duplicate analyses; the final Scott-Wilson estimations are carried out invariably in duplicate, as ample material is available at this stage.

TESTS OF METHOD.

(*a*) *With sodium β-hydroxybutyrate.*

The salt used gave practically theoretical figures on combustion [see Kennaway, 1914, 1]. The loss in the estimations on blood given below is no greater than that observed in estimations on pure solutions of the substance (*i.e.* yield of acetone about 95 % of that calculated [Schaffer and Marriot, 1913; Kennaway, 1914, 1]).

(1) 50 cc. ox blood[1] + 0·1616 g. sodium β-hydroxybutyrate = 74·4 mg. acetone
found 70·3 mg. = 94·5 %.

(2) 50 cc. ox blood + 0·1519 g. sodium β-hydroxybutyrate = 69·9 mg. acetone
found 67·3 mg. = 96·3 %.

(*b*) *With sodium aceto-acetate.*

Aceto-acetic ester was saponified in the cold with the theoretical amount of caustic soda, the solution diluted, and the aceto-acetic acid estimated by Scott-Wilson's method.

	mg. acetone in 25 cc. distillate
Calculated from weight of ester	0·8490
Found	0·8485

When such a solution is added to urine a practically quantitative yield of acetone can be obtained, whereas in the presence of blood no more than about 88 % of this amount was recovered by the same method. This is not due to any products of decomposition in the blood used, since freshly drawn blood

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[1] The amounts of acetone obtained from the blood alone in a blank estimation were of course subtracted from the result in all these experiments.

(rabbit) does not constantly give better results, although in one such experiment 93·7 % was obtained. Serum causes as much loss as does the whole blood. No relation was found between the percentage lost and the absolute amount present. The following are examples from among a considerable number of experiments.

(1) 50 cc. ox blood + sodium aceto-acetate solution = 70·7 mg. acetone
found 63·1 mg. = 89·1 %.
(2) 50 cc. ox blood + sodium aceto-acetate solution = 30·08 mg. acetone
found 26·07 mg. = 86·7 %.
(3) 50 cc. ox blood + sodium aceto-acetate solution = 30·94 mg. acetone
found 27·16 mg. = 87·8 %.
(4) Sodium aceto-acetate solution treated in same way with basic lead acetate and ammonia, but without blood
calculated 31·20 mg. acetone
found 31·22 mg. = 100 %.

(c) *With acetone.*

20 cc. acetone solution titrated by iodoform method 31·3 mg. acetone
20 cc. added to 50 cc. ox blood 30·5 mg. = 97·4 %.

Control analyses of non-diabetic bloods.

These results (Table I) serve merely as controls upon the much larger amounts of acetone bodies obtained in fatal cases of diabetes; they are not to be regarded as showing the actual amount of acetone bodies present in normal blood. It is probable that a large part of the substance obtainable from normal blood which is precipitated by Scott-Wilson's reagent is not acetone at all, for the ratio between the amounts resulting from the operations for the estimation of aceto-acetic acid and β-hydroxybutyric acid respectively is very irregular in the series. For this reason the total amounts only are given in the Table.

TABLE I. *Control analyses of non-diabetic bloods.*

		Total acetone bodies as mg. aceto-acetic acid in 100 cc. blood
Human cases, at autopsy	Heart failure	16·8
	Head injury; death 3 hours later ...	17·3
	Head injury; death 1 hour later ...	6·6
	Head injury; death 1 hour later ...	7·6
	Septicaemia	11·3
	Carcinoma of liver	4·7
	Eclampsia	3·0
	Sheep	5·0
	Sheep	8·7
	Horse (in life)	1·7
	Ox	6·2
	Ox	4·3
	Ox	4·5

TABLE II. *Amounts of acetone bodies are expressed as mg. of aceto-acetic acid in 100 cc.*

	Urine				Blood				NaHCO$_3$ given by mouth on last days of life		
	Acetone + aceto-acetic acid	β Hydroxy butyric acid	Total acetone bodies	β ratio	Acetone + aceto-acetic acid	β Hydroxy butyric acid	Total acetone bodies	β ratio	grams per day	days	Remarks
Case 5	177	306	483	63	109	170	279	61	81	1	7. vii. 13, not in coma; alveolar CO$_2$ 17·6 mm. 8. vii. 13. in coma; alveolar CO$_2$ 8·8 mm. 9. vii. 13, death in coma
„ 6	114	396	510	78	53	132	185	71	39	3	Coma; boy, aged 11
„ 7	149	522	671	78	86	165	251	66	31	3	10. iii. 13, not in coma; alveolar CO$_2$ 13·8 mm. 12. iii. 13, coma. 13. iii. 13, death in coma
„ 9 (1)	370	880	1250	70	91	152	243	62	70	5	Blood and urine (1) obtained on 30. i. 14; alveolar CO$_2$ then 17·8 mm. Conscious till death on 31. i. 14.
„ 9 (2)	185	422	607	69	96	117	213	55			Blood and urine (2) at autopsy
„ 10	229	528	757	70	106	181	287	63	62	1	6. i. 14, not in coma; alveolar CO$_2$ 15·0 mm. 7. i. 14, 5 p.m. in coma; alveolar CO$_2$ 7·2 mm. Death at 10 p.m.
„ 11	311	621	932	67	98	169	267	63	?	—	Coma
„ 12	98	192	290	66	83	125	208	60	10	2	Coma
„ 26	628	1682	2310	73	38	95	133	71	?	—	Well nourished boy, aged 22 months. Diarrhoea and vomiting from 18. vi. 14. Hyperpnoea, drowsiness. Death on 22. vi. 14. At autopsy, "status lymphaticus."
„ 29	—	—	—	—	59	40	99	40	?	—	Very drowsy for some days, but not in coma till three hours before death
„ 30	—	—	—	—	63	215	278	77	?	—	Coma
„ 31	—	—	—	—	60	101	161	63	?	—	Coma
„ 32	—	—	—	—	76	lost	—	—	?	—	Very drowsy but never comatose. Persistent vomiting. At autopsy, twisted ovarian dermoid
„ 33	—	—	—	—	36	18	54	33	?	—	Blood taken just before discharge from hospital; no signs of approaching coma. Blood sugar 0·44 %; average daily sugar in urine 340 g.
Groningen											
A.Z.	241	646	887	73	89	119	208	57	—	—	Coma. At autopsy
P.F.	491	498	989	50	94	244	338	72	—	—	„ „
L.H.	—	—	—	—	119	—	—	—	—	—	„ „
K.O.	190	—	—	—	95	—	—	—	—	—	„ „
P.K.	81	—	—	—	28	—	—	—	—	—	„ „
Marriott Case 6	—	—	—	82	49	79	128	62	—	—	"Showed signs of impending coma"

Marriot [1914] states that normal blood (of man, dog, pig and ox) contains less than 9·7 mg. total acetone bodies, reckoned as aceto-acetic acid, in 100 g. This result is in agreement with those given in Table II; in some of the pathological cases which give a higher figure an abnormal formation of acetone bodies may well have been in progress, since acetonuria may occur in almost any condition of disease.

Analyses of blood from cases of diabetes.

These are given in Table II. The cases up to No. 26 are numbered as in a previous paper [Kennaway, 1914, 2], where analyses of the urine on several days preceding death are recorded for some of them. All the cases in the table were adult diabetics, except No. 6, a diabetic boy, and No. 26, a non-diabetic infant; all the diabetics died in coma except Nos. 9, 32 and 33. All the samples of urine and blood were obtained at autopsy, except the first in case 9 and that in case 33. The estimations of alveolar CO_2 were made by Dr Poulton, to whom I am indebted for permission to include them here.

The term "β-ratio" was adopted in the previous paper to denote briefly the percentage of the total acetone bodies which is made up by β-hydroxybutyric acid, each substance being expressed in the form of acetone. If, in the absence of separate estimations of the acetone present, one regards this substance as aceto-acetic acid[1], the above percentage gives with sufficient accuracy the molecular proportions of the two acids, since their molecular weights differ by less than 2 %.

DISCUSSION OF RESULTS.

(1) Hurtley [1916] and Hurtley and Trevan [1916] have brought forward evidence to show that aceto-acetic acid has a specific toxic action, apart from its acid character, and that β-hydroxybutyric acid is a much less toxic substance both because it is a weaker acid (by eight times), and because it lacks the $C.OH = CH$ group, which is held to be the cause of the special toxicity of aceto-acetates and of other compounds having this group (sodium salicylate, acetylacetone). One may examine the figures given in Table II to see if they have any application to this view. If one excludes the non-fatal

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[1] The quantity of acetone may be by no means unimportant in this connection; in Marriot's Case 6 (see Table II) it was found to be 63 % of the acetone + aceto-acetic acid. In such estimations, however, there must always be uncertainty as to the amount of decomposition of the acid to acetone which has occurred in vitro.

case No. 33 and the non-diabetic case No. 26 the amounts of acetone bodies show the following range (see also Fig. 1):

	Minimum		Maximum		Ratio
Aceto-acetic acid, mg.	53	(Case 6)	109	(Case 5)	1 : 2
β-Hydroxybutyric acid, mg.	40	(Case 29)	215	(Case 30)	1 : 5·5

Marriot's case (in life) gave a slightly lower figure (49 mg.) for aceto-acetic acid; two out of Geelmuyden's seven results are above these limits (119 mg. aceto-acetic acid in L.H. and 244 mg. β-hydroxybutyric acid in P. F.), and one is considerably lower (28 mg. aceto-acetic acid in P.K.).

In cases 29, 30 and 31 one has three instances in which the amounts of aceto-acetic acid are almost the same (59, 63, and 60 mg.) while those of β-hydroxybutyric acid (40, 215 and 101 mg.) include the maximum and minimum given in the summary above. Fig. 1 illustrates the variations which occur in the relative amounts of the two acids.

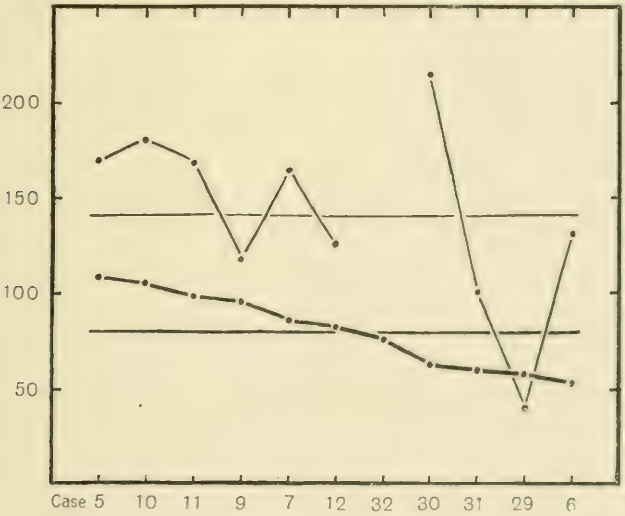

Fig. 1. Cases of Diabetes, at autopsy.

Ordinates represent acetone bodies, expressed as mg. aceto-acetic acid, in 100 cc. blood.
Upper series, β-hydroxybutyric acid.
Lower series, acetone + aceto-acetic acid; these are placed in order of magnitude
The horizontal lines represent the respective means of the two series.

If one is to derive from these results any evidence as to the relative toxicity of the two acids, it is certainly suggestive that the range of the amounts present at death is much smaller in the case of the aceto-acetic acid: differences in individual susceptibility might perhaps cause variations of 100 % in the lethal dose of a substance, but would be unlikely to explain the range of 550 % seen in the amounts of β-hydroxybutyric acid. It might be said that the large differences in the amounts of the latter acid tolerated before the fatal issue were due to differences in the amounts of alkali administered, but the data on this point given in Table II do not show any such influence; and furthermore, this factor should lead one to look for as large variations in the amounts of aceto-acetic acid, since this is an acid eight times as strong as the other.

It is noteworthy that in the 24 hours preceding death in case 9, the β-hydroxybutyric acid underwent a distinct fall, and the aceto-acetic acid a slight increase, in amount; this might be attributed [Hurtley, 1916] to failure of the protective activity of the liver in converting the latter acid into the former. The recent experiments of Wilder [1917] show the conversion of aceto-acetic acid to β-hydroxybutyric acid (in the dog).

These results are then compatible with the view that aceto-acetic acid has a specific toxic action and are perhaps as favourable to it as can be expected from their nature; single observations cannot throw much light upon the course of a progressive intoxication. A much larger series of observations, made in each case before and during coma, and after death, together with measurements of the hydrogen ion concentration in the blood and full daily analyses of the urine, and records of the administration of alkali, are required to give any definite information on this question.

(2) Although, as was pointed out above, there is no constant relationship between the amounts of β-hydroxybutyric acid and aceto-acetic acid in the blood, the most frequently occurring proportion in this series is certainly such that there are between 1·5 and 2 molecules of the former acid to one molecule of the latter, for the β-ratio in seven of the ten fatal diabetic cases lies between 60 and 66. Case 33, in which there were no signs of approaching coma, shows a much smaller amount of acetone bodies, together with a much lower β-ratio (33), than do any of the other cases. The high proportion of aceto-acetic acid in such a case is to be expected from the results obtained with a considerable number of urines from cases of diabetes and of other conditions of acidosis [Kennaway, 1914, 2]. When the amount of the two acids is low, the amount of aceto-acetic acid is relatively high; it falls as the severity of the case increases, and tends to rise again on the approach of coma [Hurtley, 1916]. If one adopts the view that aceto-acetic acid has a special toxicity the explanation of this series of changes would seem to be as follows: if the total amount of acids is sufficiently small, the presence of a high proportion of aceto-acetic acid is of no great importance, and the β-ratio remains low; as the absolute amount rises, a protective mechanism

for the conversion of aceto-acetic into β-hydroxybutyric acid is called into increased activity, and the β-ratio rises; while the final increase in the proportion of aceto-acetic acid would indicate the failure of this mechanism (see Table II, first and second samples in case 9).

The figures for the β-ratio given in Table II show that the proportion of β-hydroxybutyric to aceto-acetic acid is always higher in the urine than in the blood[1]. One might be inclined to attribute this difference to a final accumulation of aceto-acetic acid in the blood before death, after the bulk of the urine found in the bladder at autopsy had been secreted; but the samples of blood and urine obtained from case 9 in life show the same difference. The explanation may be that the kidney receives the blood within a few seconds of its passage through the lung, where acetone has escaped; whereas the venous blood, after its slower passage through the systemic capillaries and veins, will have developed a fresh quantity of this substance.

(3) The data given in Table II enable one to form some quantitative idea of the strain which the production of these quantities of acids must place upon the maintenance of neutrality in the body.

(*a*) Suppose that a patient such as No. 5, with acetone bodies equivalent to 280 mg. aceto-acetic acid in 100 cc. blood, weighed 70 kilos and contained 3·5 litres of blood; the amount of acid in the whole blood would be 9·8 g. According to Schmidt's [1850][2] analyses, the plasma of 1000 g. of human blood contains 1·66 g. sodium. 9·8 g. aceto-acetic acid is equivalent to 2·21 g. sodium, which would be contained in the plasma of 1330 g. or 1250 cc. of blood, which is more than one-third of the whole blood in this case. Of course other bases, especially ammonia and calcium, are available as well; but some calculation of this sort may serve to illustrate the difficulties under which neutrality is maintained, or nearly maintained [Poulton, 1915] in severe diabetes.

The concentration of acetone bodies in this case (No. 5) if expressed as aceto-acetic acid, and regarded as an aqueous solution of the free acid, is nearly that of a 0·03 N solution.

(*b*) In case 9, the blood in life contained 243 mg. acetone bodies as aceto-acetic acid in 100 cc.; this is equivalent to 52 cc. CO_2 from $NaHCO_3$ at 0° and 760 mm., *i.e.* to the whole amount of CO_2 obtainable from this volume of normal blood. The alveolar CO_2 tension at the time when this blood was withdrawn was found by Dr Poulton to be 17·8 mm.; if the curve given by Christiansen, Douglas, and Haldane [1914] showing the CO_2 content of blood in relation to CO_2 tension were applicable to abnormal bloods, one could obtain from these data the amount of CO_2 displaced from the blood by this known amount of abnormal acid, which would be of considerable interest.

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[1] Geelmuyden's case P.F. is an exception.

[2] These seem to be the most modern full analyses of human blood, and are those which appear in most text-books. Schmidt's tables give 1·66 g. sodium in the 487 g. plasma of 1000 g. of human blood; Arronet and Warnach [1887, 1888] found 0·344 g. sodium in 100 g. of human serum. The results on plasma and serum therefore agree very closely ($0·344 \times 4·87 = 1·67$).

(*c*) 243 mg. aceto-acetic acid (see case 9) is equivalent in molecular proportions to 214 mg. lactic acid; this amount of lactic acid, when added to 100 cc. blood, is sufficient to produce the same effect upon the dissociation constant of haemoglobin, as expressed by K, as is produced by the whole normal tension of CO_2 [see Barcroft, 1914, Fig. 111].

(*d*) The data given for the first eight cases in the table illustrate the part played by the kidney in the maintenance of neutrality. In this series, the mean concentration of acetone bodies in the urine (868 mg. %) is nearly four times that in the blood (230 mg. %)[1]. In case 9, the complete 24 hours' urine could not be obtained; but if its volume had been 3 litres, a common amount in such cases, and the patient's blood-volume 3·5 litres, the protective action of the kidney would have been equivalent to freeing the whole blood of acetone bodies between four and five times in the 24 hours.

In the non-diabetic case 26, the very high concentration of acetone bodies in the urine (more than seventeen times that in the blood) is noteworthy; it is no doubt due to excessive loss of water from the alimentary canal. The figures emphasise the need for a very abundant supply of fluid, given by any available method, in such cases.

(*e*) Hurtley [1916] points out that if the intoxication in severe diabetes were no more than a simple acid poisoning, such as can be produced experimentally in animals, treatment by alkalis should be more successful than it is found to be in practice. As much as 3 ounces (93 g.) of sodium bicarbonate[2]

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[1] Dr Poulton has pointed out to me that the data given in the previous paper [Kennaway, 1914, 2] for case 5, in which the percentage of acetone bodies in the urine at death (483 mg.) is only 1·9 times that in the blood (279 mg.), show a fall in the concentration of acetone bodies removed by the kidney, as well as in the alveolar CO_2 tension, the latter change being no doubt due to the retention of increasing amounts of acid.

	July 2	3	4	5	7	8	9 (day of death)	at autopsy
Total acetone bodies of urine, as gms. aceto-acetic acid per cent.	1·53	1·83	1·42	1·60	—	0·857	1·04	0·483
Alveolar CO_2, mms. Hg.	27·0	—	26·9	24·5	17·6	8·8	—	—

[2] Such large amounts of sodium, when given without counteracting ions, may not be altogether harmless. Locke's fluid for perfusion of the mammalian heart contains Na, K, and Ca approximately in the proportions Na : K : Ca/100 : 6 : 2·5. These proportions of the three kations are

is sometimes given in a day; this is sufficient to neutralise 113 g. of the acids, which is almost equal to the amount which might be present in the whole body of a case such as No. 9, supposing the acids to be distributed uniformly throughout the blood and other body fluids (about 50 litres in a person weighing 70 kilos). As a matter of fact, this case (No. 9) did receive on each of the five days preceding death an amount of $NaHCO_3$ (70 g.) sufficient to neutralise the abnormal acids in 35 litres of blood having the composition found at this time; this would be quite 10 times the blood volume of the patient. Moreover, it is exceptional for a diabetic to produce even as much as 50 g. of the acids, as indicated by the output in the urine, in a day. There can, of course, be no doubt that the body in such cases has urgent need of the assistance of alkaline remedies, but the disappointing nature of the results certainly indicates that some additional factor, other than the simple neutralisation of acid, is involved.

The intravenous or subcutaneous injections of alkali given when coma is impending or has supervened have as a rule only a transient beneficial effect. 350 cc. of the 2 % solution of sodium bicarbonate commonly employed would suffice to neutralise an amount of the acids equal to that (8 or 9 g.) present in the whole blood-volume of a patient such as No. 9; but since, as was pointed out above, the blood may be as it were refilled with acid four or five times daily, no persistent effect is to be expected without some method of continuous injection.

SUMMARY.

The concentration of acetone bodies in the blood in diabetic coma may be equivalent, when reckoned as aceto-acetic acid, to that of a 0·03 N solution: this amount is sufficient to combine with more than one-third of the sodium of the plasma. The amounts of aceto-acetic acid show less variation in the series of cases than do those of β-hydroxybutyric acid.

Methods for the estimation of acetone and aceto-acetic acid are as a rule tested with acetone only; this is quite inadequate, as the recovery of aceto-

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contained, with sufficient accuracy, in a mixture of 50 parts $NaHCO_3$, 2 parts $KHCO_3$, and 1 part $CaCl_2$; the official $KHCO_3$ is here substituted for the KCl of Locke's solution. This simple ratio could be applied in the prescription of any desired amount of $NaHCO_2$ for administration by the mouth, *e.g.*:

Sodium Bicarbonate	150 grains
Potassium Bicarbonate	6 ,,
Calcium Chloride	3 ,,

This solution is of course turbid from the precipitation of $CaCO_3$, but the $CaCl_2$ might be given at a different time from the rest.

acetic acid is liable to an error which does not occur in the case of either acetone or β-hydroxybutyric acid.

The expenses of this investigation were defrayed by a grant from the Science Committee of the British Medical Association.

I am indebted to Dr Turnbull, of the London Hospital, for the material from three of the cases of diabetic coma.

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XI. THE ANTI-SCORBUTIC VALUE OF COW'S MILK[1].

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(*Received February 15th, 1918.*)

INTRODUCTION.

THE experiments described in the following paper form a small group out of a much larger scheme. The main scheme was planned in order to obtain a comparison between the anti-scorbutic values of a number of foodstuffs, in response to war-time requirements, both civil and military, within the British Empire. Some of the preliminary results have already been published [Chick and Hume 1917; Chick, Hume and Skelton 1918] owing to urgent practical need for their application on active service and at home, and it was intended that a detailed report of the whole work should appear later, when the investigation was complete.

We believe, however, that any contribution to the study of the nutritional value of milk may be of immediate practical value in connection with the problems of infant feeding, particularly at the present time. The following experiments are by no means complete or exhaustive, but, though few in number, they form a complete and concordant series. Work of this character involves great labour and the experiments with milk have had temporarily to be abandoned to permit time and attention to be devoted to other, equally important foodstuffs. It has therefore seemed wisest not to delay publication of the results already obtained.

It has been usual to regard fresh cow's milk as a substance possessing valuable anti-scorbutic properties and capable of preventing scurvy, if present in a diet in even moderate amount. This view is held by many authorities on infant feeding. There does not, however, appear to be any sound basis for such an opinion and though direct evidence upon this point is scanty, what there is, is opposed to such a view. Curran [1847] in his description of the scurvy epidemic in Dublin in 1847, cites more than 80 cases among Union inmates, all of whom had received one pint of milk daily for at least six months before falling ill. The diet in the Unions, however, was deficient in fresh meat and vegetables, and potatoes had been scarce, owing to the potato famine.

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[1] A short summary of this paper appeared in the *Lancet*, Jan. 5th, 1918.

More recent evidence upon this point is derived from careful experience of infant feeding. The observations of Hess and Fish [1914], Hess [1916], Miller [1917] and others also suggest that cow's milk is a food-stuff comparatively poor in anti-scurvy substances. They indicate further that many methods of preparation of food for the use of infants, such as heating, drying, etc., tend to diminish the small amount of these valuable constituents originally present, with a distinct resultant risk of causing slight and incipient infantile scurvy. The results of our experimental work are in accord with the observations of these workers, which deserve wide publication and will be referred to in detail later, see page 150.

Experimental.

From the close analogy in etiology, symptoms, method of cure and postmortem appearances in case of death, guinea-pig scurvy has generally been accepted as the equivalent of human scurvy by those having experience of both conditions. The guinea-pig has been selected for the study of experimental scurvy on grounds of general convenience and because this animal is particularly susceptible to the disease. If all fresh vegetable food is removed from a young, growing guinea-pig on a diet of grain and water, scurvy symptoms will appear in about 20 days and death will occur in about a month (see Table I and Chart 1). The anti-scorbutic value of various foodstuffs can be estimated by adding these in varying amounts to the scurvy diet of grain and water.

Guinea-pigs were used by Holst and Frölich [1912] in their classic work on experimental scurvy and by Frölich [1912] who specially investigated cow's milk. He found that these animals could be protected from scurvy by an exclusive diet of fresh milk and that raw milk was a better diet than heated milk. It is difficult to interpret his experiments fully, as no indication is given of the quantities consumed.

Funk [1913] states that 50 cc. of fresh unboiled milk daily, in addition to oats, prevented loss of weight and onset of scurvy in guinea-pigs, a result which we have not succeeded in confirming. His experiments are not, as far as we are aware, set out anywhere in detail.

Jackson and Moore [1916] found that of guinea-pigs fed on pasteurised milk *ad libitum*, 5 remained well out of 32; on raw milk, out of 9, all died. McCollum and Pitz [1917] fed guinea-pigs on oats and fresh cow's milk *ad libitum*, and found that some survived and some died. In both these researches no figures are given for the amounts of milk actually consumed by the animals. The present work is therefore the first in which a special effort has been made to include the quantitative factor.

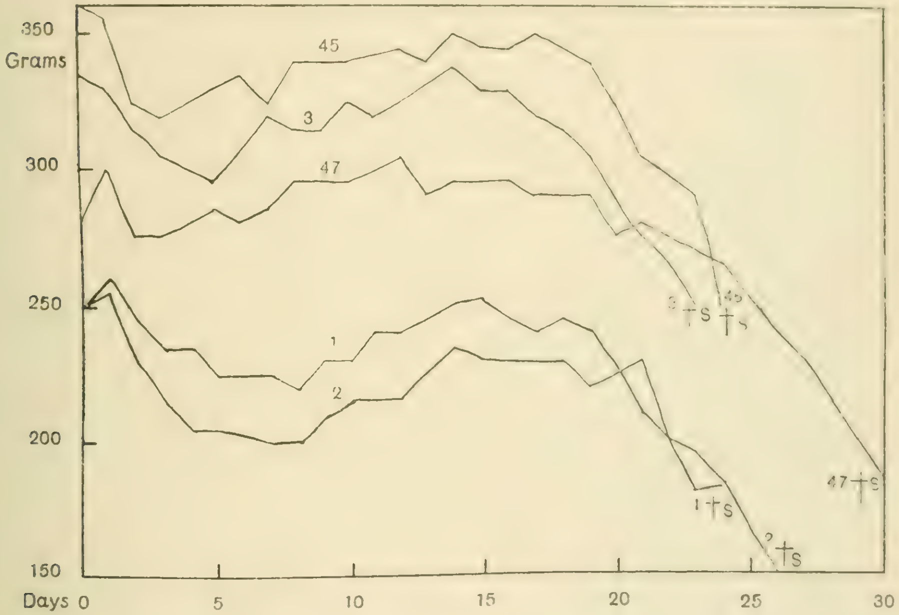

Chart 1. Weight curve of young guinea-pigs upon a diet of oats and bran (*ad lib.*) and water, see Table I. †s signifies death from scurvy.

The general course of events in an unprotected guinea-pig, when grain and water alone are given, is seen on reference to Chart 1 and Table I. The details of 5 young guinea-pigs fed upon oats, bran and water *ad libitum* show a considerable degree of consistency. The initial weight, 250–360 grams, is maintained, but barely exceeded, for a period of 15 to 18 days (see Chart 1), after which it gradually falls till at death the loss is 25–30 % of the original. Definite scurvy symptoms were noted after periods of 17–22 days; and death from scurvy occurred between the 23rd and 30th day.

TABLE I. *Development of Scurvy in Young Guinea-pigs upon a diet of Grain and Water.*

No. of experiment	No. of animal	Diet	Weight of animal, g.				Length of experiment, days	Day on which symptoms of scurvy were first noted	Result	
			At beginning	Highest reached	At end	Change in weight, %				
1	1	Oats, wheaten bran, water, *ad lib.**	250	260	182	−37	24	uncertain	Death from scurvy,	24th day
2	2	do.	250	250	150	−40	26	22	do.	26th ,,
3	3	,,	335	338	250	−25	23	17	,,	23rd ,,
4	45	,,	360	360	250	−31	24	21	,,	24th ,,
5	47	,,	280	300	185	−34	30	17	,,	30th ,,

* In health the animals consumed about 30–50 g. oats and bran daily; when sickening with scurvy the amount decreased to 10–20 g. or less.

The use of a basal diet of oats, bran and water is open to the criticism that it is deficient in another accessory food-factor besides the anti-scorbutic one, *i.e.* the "Fat Soluble A" of McCollum and others, and that therefore any experiments performed with it will be subject to two limiting factors instead of one. We have therefore made it our general custom in studying experimental scurvy to add to the diet of oats and bran, 60 cc. a day of milk autoclaved at 120° C. for an hour: of this from 30 to 60 cc. were usually consumed. We have not found that this addition delays the onset of scurvy symptoms but it does alter the character of the weight curve. If Chart 1 (oats, bran and water) be compared with Chart 2 (oats, bran and autoclaved milk), it is seen that while on the first diet, weight is maintained till the first onset of scurvy symptoms, on the second diet there is a steady gain in weight for about the same period.

This effect of milk in maintaining growth after it has been deprived of its anti-scorbutic vitamine is interesting in view of the results obtained by Hopkins [1912] and later by McCollum and Davis [1913] and by Osborne and Mendel [1913] in their experimental work on growth. The accessory growth factor, termed "Fat Soluble A," of which one important source is butter fat, would appear to be operating in our autoclaved milk, and to be distinct from the anti-scorbutic factor.

Further experiments are in progress in order to test whether this explanation is correct or whether the influence of the milk ration in promoting growth is, in this case, due simply to its content of fat, protein and salts.

These American investigators, working with young rats, have arrived at the conclusion that, in addition to adequate provision of protein, carbohydrate, fat and salts, for nutritive purposes two accessory factors are required, if satisfactory growth is to be maintained. The first is termed "Fat Soluble A" [McCollum and Kennedy 1916], and is to be found among other sources in butter fat and egg fat [McCollum and Davis 1913], in beef fat [Osborne and Mendel, 1915], in codliver-oil [Osborne and Mendel, 1914], and in some green leaves [McCollum, Simmonds and Pitz, 1918]. The second "Water Soluble B" [McCollum and Kennedy, 1916], of which rich deposits are found in the germ of cereals [McCollum and Davis, 1915] and in yeast [Funk and Macallum, 1915], is considered [McCollum and Kennedy, 1916] to be identical with the accessory factor (anti-beri-beri or anti-neuritic vitamine) protecting from avian polyneuritis and human beri-beri. McCollum and his co-workers do not admit that there is an additional factor protecting from scurvy. It is however possible that had their work been carried out with another experimental animal they might have reached another conclusion. It is well known that the rat is insusceptible to scurvy, and it may well be that it requires only a small amount of the anti-scorbutic factor and that sufficient traces may remain in the substances of animal and plant origin which supply the two "growth" factors.

Our work leaves us in no doubt that, in the case of guinea-pigs and man, an accessory anti-scorbutic factor is required to maintain health. Although in many cases growth will cease in young animals if this anti-scorbutic factor is omitted from the diet, it does not appear likely from a study of their respective distributions that this anti-scurvy vitamine is identical with either of the two growth factors described by the American workers. In our own experiments, the anti-beri-beri vitamine (probably identical with the growth factor "Water Soluble B") is present in abundance in the ample ration provided of whole oats and wheaten bran. The second growth factor, "Fat Soluble A," may be operating in our milk ration (even when strongly heated) and is distinct from the anti-scurvy accessory factor.

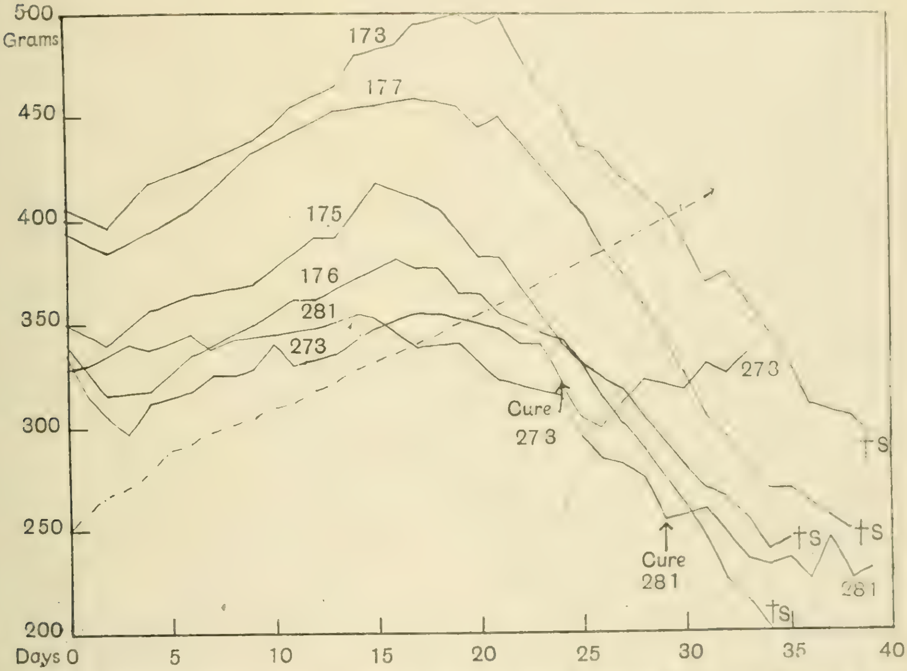

Chart 2. Weight curve of young guinea-pigs upon a diet of oats and bran (*ad lib.*) and autoclaved (1 hour at 120° C.) cow's milk up to 60 cc. daily, see Table II, Expts 1-6.

·—·—·— signifies normal weight curve upon a diet of oats and bran and fresh cabbage *ad lib.*

†s signifies death from scurvy.

↑ Cure signifies cure begun with fruit juice.

It is necessary to give a short description of what is meant in the Tables by "scurvy symptoms." The first symptom to be observed is soreness of joints and limbs, more especially of shoulders and knees, so that the animal squeaks when pressure is applied to these places. Some animals squeak when

TABLE II. *Influence of addition of (a) strongly heated (120° C. for one hour) milk and (b) dried milk, upon Scurvy in Guinea-pigs upon diet of Oats and Bran compared with (c) other anti-scorbutic materials.*

		Diet			Weight of animal, g.						
			Average daily ration of milk, cc.								
No. of experiment	No. of animal	Average daily ration of oats and bran	(a) *Autoclaved milk* 1st 21 days	Later	At beginning	Highest reached	At end	Change in weight, %	Length of experiment, days	Day on which symptoms of scurvy were first noted	Result
1	173	oats and bran, *ad lib.*	60	55	405	500	298	−26	40	21	Death from scurvy, 40th day
2	175	,, ,,	45	20	347	418	200	−43	34	21	,, ,, 34th ,,
3	176	,, ,,	41	40	340	382	245	−28	35	21	,, ,, 35th ,,
4	177	,, ,,	47	32	395	458	240	−39	37	18	,, ,, 37th ,,
5	273	,, ,,	45	34	339	355	320	− 6	24	15	In condition of acute scurvy, 24th day, cure with lemon juice started
6	281	,, ,,	42	22	330	356	255	−23	29	not noticed	"Cure" with fresh lime and fresh orange juice started 29th day
			(b) *Dried milk*								
7	163	,, ,,	60*		346	373	237	−32	34	28	Death from scurvy, 34th day
8	162	,, ,,	76*		350	395	350	0	23	21	"Cure" with preserved lemon juice started 23rd day
		(c) *Other anti-scorbutic materials*	autoclaved milk								
9	440	*ad lib.* + 2·5 g. fresh cabbage	ca. 60		320	increasing to	572	+79	95	—	Good health
10	423	*ad lib.* + 5 g. fresh cabbage	ca. 60		299	,,	565	+89	92	—	,, ,,
11	239	*ad lib.* + 3 cc. fresh orange juice	ca. 60		365	,,	598	+64	93	—	,, ,,
12	103	*ad lib.* + 10 cc. fresh raw beef juice	0		350	350	240	−31	23	15	Death from scurvy, 23rd day

* Reckoned as the equivalent of ordinary milk.

handled under any circumstances, and when in perfect health; but if they are examined regularly from the beginning of the experiment, it is possible to distinguish those which are feeling pain. The presence of painful members is also shown by the assumption of what we have called the "scurvy position," which seems to indicate haemorrhage and consequent discomfort in the muscles of the limbs. The animal rests on its side, and the painful leg is held off the ground and may be seen twitching. A second attitude, which we have called the "face-ache position," is also indicative of scurvy in young guinea-pigs; the animal lies curled up with the side of its face pressed on the floor of the cage. This is a frequent attitude in adult guinea-pigs when in normal health, but we have never seen a young animal adopt it except when ill with scurvy. It seems to indicate haemorrhage of the jaw, with soreness and looseness of the teeth. The state of the molar teeth and of the whole gums cannot be inspected during life, and it is only possible to judge of the condition by the greater or less capacity for eating, and by the assumption of the "face-ache position."

Post-mortem appearances. The macroscopic signs of scurvy in guinea-pigs consist mainly of haemorrhage and bone lesions and have been well described by Holst and Frölich [1907, 1912]. It may be convenient to recapitulate them here.

The haemorrhage may occur in almost any situation; for example, in the subcutaneous connective tissue, especially in the region of the axilla or groin, and over the ribs, or among the muscles, more frequently of the upper and lower limbs, and less often in the intercostal muscles. In chronic cases of long standing, the whole musculature appears darker in colour than in the normal animal. Haemorrhages in the walls of the viscera are also frequent; in these cases blood is often passed with the faeces during life and death occurs suddenly.

The changes in the bony tissues are characteristic, and are conveniently observed in the knee joints. In a severe case these are very much swollen, and on dissection the tibial epiphysis (or more rarely the femoral) is found to be disorganised and often broken across; the condyles can best be described as papery, the shafts of the long bones are brittle and can be easily broken with forceps, and the marrow is much darker than the normal. Periosteal haemorrhages, which form so marked a feature of infantile scurvy, are seen in comparatively few cases. The jaws and teeth are usually profoundly affected, the teeth become brittle and loose, and the jaw is easily fractured on pressure.

Typical changes take place in the costo-chondral junctions. They become swollen, and exhibit a transverse yellow bar and are frequently surrounded with haemorrhages in the adjoining muscles. These appearances correspond with the complete disorganisation of the bone-cartilage junction, and with disappearance of the cartilaginous trabeculae and of the rows of cartilaginous growing cells. In many cases the bone is found to be completely fractured and there is great proliferation of connective tissue across the junction.

Experiments with fresh Cow's Milk.

Group I.

The experiments with fresh milk fall into two groups.

The first group of experiments consists of those in which not more than 30 to 50 cc. of fresh milk were presented to each animal daily, together with an *ad libitum* (measured) ration of oats and bran. The larger quantity of milk was given under the impression that we should protect our animals from scurvy by such a ration [Funk, 1913]. This, however, proved not to be the case, and in every instance acute scurvy was the result.

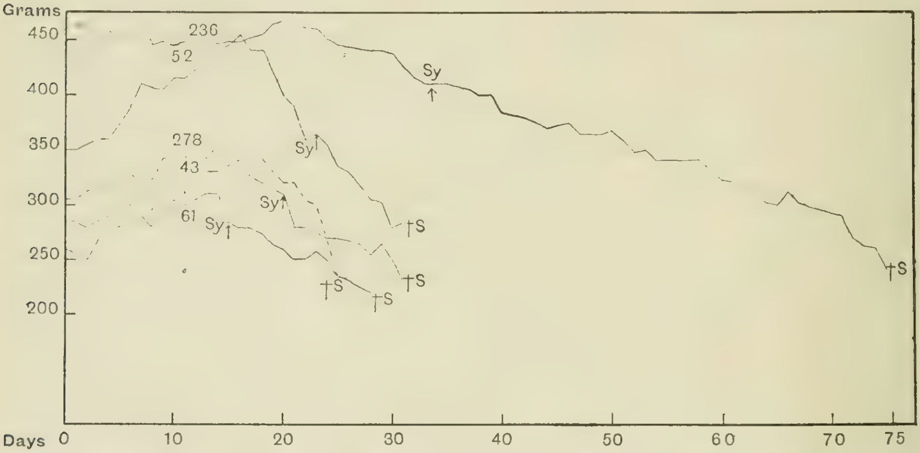

Chart 3. Weight curve of young guinea-pigs upon a diet of oats and bran (*ad lib.*) and fresh cow's milk 30–50 cc. daily, see Table III. In case of No. 236, the daily ration of 50 cc. was maintained continuously by hand-feeding.

Sy ↑ signifies scurvy symptoms noted. †s signifies death from scurvy.

·—·—·— signifies 1 cc. liquid paraffin given every second day.

The details concerning the 8 animals in this group are set out in Table III and Chart 3, and present a very uniform result. All (with the exception of No. 236, described later) developed scurvy symptoms in 15 to 28 days, an average time not very much longer than when the milk was omitted (see Table I). In cases where no treatment was essayed, the symptoms became progressively severer, great loss of weight occurred, and the animals became moribund about 10 days later. They were usually killed by chloroform at this stage (Nos. 1, 2, 4, 7, Table III).

In the case of other animals in this group fresh fruit juices were given after definite scurvy symptoms had been observed, with the result that successful cures were obtained. The animals, after a short interval, began to

TABLE III. *Influence of Daily Ration of 30–50 cc. fresh Cow's Milk upon Scurvy in Guinea-pigs. The diet consisted otherwise of Oats and Bran* ad libitum, *the amount of which consumed varied between 20 and 40 g. daily.*

No. of experiment	No. of animal	Daily ration of milk offered, cc.	Weight of animal, g. At beginning	Weight of animal, g. Highest reached	Weight of animal, g. At end	Weight of animal, g. Change in weight, %	Length of experiment, days	Day on which symptoms of scurvy were first noted	Result	Post-mortem examination result
1	43	30	285	340	215	−25	31	20	Very ill, killed 31st day	Scurvy
2	44	30	290	295	205	−29	29	not noticed	Moribund, killed 29th day	,,
3	51	50	320	375	260	−19	31	20 slight 27 severe	"Cure" 5 cc. fresh orange juice daily started 31st day. Killed 77th day in fair health. Weight = 420 g.	Traces of old scurvy
4	52	50	350	450	285	−18	31	28	Very ill, intestinal haemorrhage. Killed 31st day	Scurvy
5	53	50	355	410	290	−18	31	20	"Cure" 5–10 cc. fresh orange juice daily started 31st day. Splendid condition 90th day. Weight = 470 g. Used for breeding	
6	60	50	280	385	340	+21	17	18	"Cure" 10 cc. fresh orange juice daily, 18th day. Killed 63rd day, in fair health. Weight = 450 g.	Traces of old scurvy
7	61	50	260	310	220	−15	30	15	Death from scurvy, 30th day	Scurvy
8	278	60 + 1 cc. liquid paraffin each second day	305	355	237	−22	25	not noticed	,, ,, 25th ,,	,,
9	277	,,	318	335	295	−7	24	15	"Cure" attempted with lime juice beginning 24th day	Traces of old scurvy

put on weight, the teeth, if loose, began to tighten up, and very slowly the soreness of the joints began to improve. The animals were killed after varying periods of time, and, although in good health, traces of the old scurvy were demonstrable even when some months had elapsed since the date of acute symptoms. These traces consisted in survival of the bone changes in acute scurvy, such as swellings at costo-chondral junctions, and malformation of leg bones with bony outgrowths due to partial or complete separation of the tibia at the upper epiphysis and subsequent joining up again of the fractured bone in the wrong position. Disturbance in the position of the teeth was also sometimes noted.

No animal in this group failed to develop scurvy. The ration of milk offered (*Expts.* 1, 2, 30 cc.; *Expts.* 3 to 9, 50 to 60 cc.), was usually consumed as long as the animal remained well; with the onset of scurvy symptoms the amount of milk taken decreased until the end of the experiment, 10 to 15 days later. The supply of anti-scurvy accessory substance is here so deficient that individual idiosyncracy and power of resistance play an insignificant part. In our experience no guinea-pig can remain in health over a long period upon a diet of oats and bran, to which as little as 30 to 50 cc. of fresh milk daily is added.

GROUP II.

In the second group of experiments, the amounts of fresh milk consumed were greater, viz.—from 50 cc. upwards.

This group (see Table IV, Charts 3 (No. 236) and 4) contains few animals, but the nature of the work made this unavoidable. Throughout the scurvy investigation in the Institute the rule has been that the animals are not left to the care of the laboratory servants, but are handled and weighed by the worker in charge, who also measures the amount actually consumed of each constituent of the diet. We have found that this is the only way in which a really satisfactory knowledge of the working of the experiment can be gained, although the amount of the daily routine is greatly multiplied. In addition to these precautions, it was often necessary, with the animals in the second group, to reinforce with hand-feeding, in order that the standard of milk consumption should be kept up. It is not, however, possible to give more than about 50 cc. of milk by hand during the day and, unless the animal also cooperates heartily in the experiment during the night, the total daily consumption does not rise above 70–80 cc.

The ration of oats and bran was cut down to a minimum, 10 g. in order

TABLE IV. *Influence of Daily Ration of 50–130 cc. fresh Cow's Milk upon Guinea-pig Scurvy.*

No. of experiment	No. of animal	Average daily ration of milk consumed, cc.	*Ration of oats and bran, g.	Weight of animal, g. At beginning	Weight of animal, g. Highest reached	Weight of animal, g. At end	Weight of animal, g. Change in weight, %	Length of experiment, days	Day on which symptoms of scurvy were first noted	Result	Post-mortem examination results
1	236	50†	*ad lib.*	451	469	240	−47	75	33	Very ill, scurvy. Killed 75th day	Scurvy
2	232	65	*ad lib.*	467	507	382	−18	39	28	Accidentally killed 39th day	,,
3	234	1st period, 14 days 68 2nd ,, 32 ,, 57 3rd ,, 34 ,, 73	10	275	340	268	−2	83	21	Moribund, killed 83rd day	,,
4	242	1st period, 44 days 86 2nd ,, 21 ,, 57 3rd ,, 24 ,, 61	10	453	490	300	−34	91	45	Death from scurvy, 91st day	,,
5	243	85	*ad lib.*	480	gradually increasing to	555	+16	24+	never	Good health, birth of 2 young on 24th day	No scurvy
6	235	1st period, 22 days 84 2nd ,, 31 ,, 132 3rd ,, 36 ,, 129 4th ,, 30 ,, 124	10	260	,,	586	+125	113+	never	Good health	—

* In health the animals fed *ad lib.* consumed 30–50 g. daily; when sickening with scurvy the amount decreased to 10–20 g. or less.

† This animal did not voluntarily take as much as 50 cc. daily, the amount not consumed was fed by hand, so that the daily consumption was regularly 50 cc.

that a sufficient volume of milk could be accommodated; we also found that a moderately high temperature of the laboratory was helpful in increasing thirst. In all the experiments the milk was given twice daily. At first it was ordinary dairy milk, but much trouble was encountered through souring during the night, after which most animals refused to touch it. For a short time boracic acid (0·5 g. per litre) was added to the night milk to preserve it, but this did not improve the animals' appetite. For the latter part of the time, in the experiments of this group, very pure, specially delivered, country milk was used. No further trouble was experienced with souring.

The usual procedure was to offer 50 cc. of milk in the morning which had to be consumed during the day, with the help of some hand-feeding, if necessary. At night, a measured amount, graded to the particular appetite of the animal in question, was offered, and the residue was carefully measured next morning.

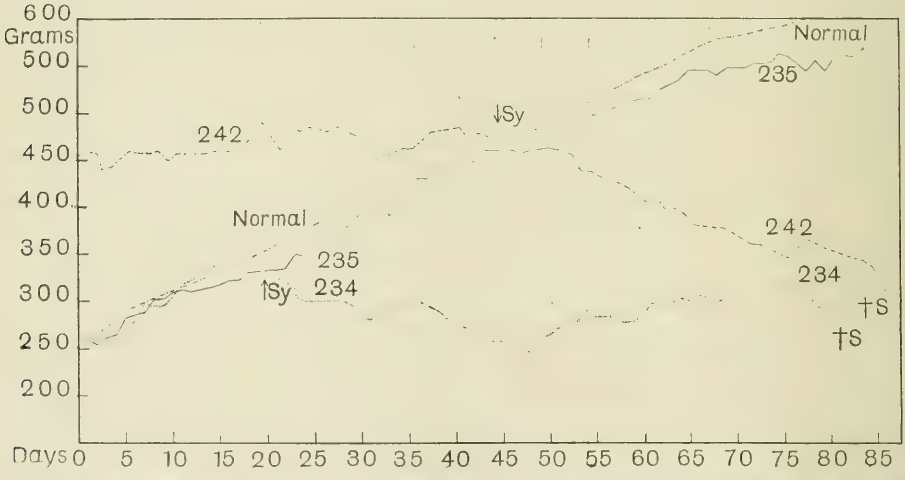

Chart 4. Weight curves of young guinea-pigs upon a diet of oats, bran and fresh cow's milk 50–130 cc. daily, see Table IV: No. 234, 60–80 cc., No. 242, ca 80 cc., No. 235, 80–130 cc.

·—·—·— signifies normal weight curve upon a diet of oats, bran and fresh cabbage *ad lib.*

†S signifies death from scurvy.

↓ Sy signifies scurvy symptoms observed.

The details concerning animals in Group II are set forth in Table IV, and the weight charts of three typical cases are shown in Chart 4; the animals themselves, however, deserve individual notice.

No. 236 (initial weight 450 g.), offers an interesting contrast with the animals of Group I, and its weight chart is set out in Chart 3, for purposes of comparison with the animals of that group. This animal consumed exactly 50 cc.

of milk daily throughout the experiment. Incipient symptoms of scurvy appeared about the 33rd day, and a gradual decline of weight set in, but the animal did not become acutely ill until the 75th day. This is in marked contrast with the other animals in Group I, and is attributable to the fact that the daily ration of 50 cc. fresh milk did not fall off when the animal became ill, but was fed daily to it by hand.

The weight curve of No. 236 we regard as characteristic of partial protection against scurvy. The anti-scorbutic accessory factor is present in the diet but in insufficient quantity and the life of the animal is prolonged, though it ultimately succumbs with symptoms of scurvy of long standing.

No. 232 (initial weight 467 g.), consumed an average of 65 cc. milk, and did well for a time. About the 22nd day it showed symptoms of scurvy, and its weight fell rather fast. At this period even with hand feeding it was occasionally receiving less than 50 cc. of milk daily. About the 30th day it became very thirsty and consumed 70 to 80 cc. daily and the weight improved. Unfortunately the animal became enfeebled with an attack of diarrhoea, its daily consumption of milk reached 100 to 110 cc., but in its eagerness it unfortunately fell into its milk pot and was drowned.

No. 234 (initial weight 275 g.). The average daily consumption of milk varied from 68 cc. for the first two weeks, to 57 cc. for the next four weeks, and 73 cc. for the last five weeks. The weight of the animal was well maintained throughout the 83 days of the experiment, but symptoms of scurvy were apparent from the 21st day.

No. 242 (initial weight 453 g.). The average daily ration of milk was at first greater than in case of 234, viz., about 86 cc.; later it fell below it. On the whole, however, the result corresponded very nearly with that of No. 234 though the animal was much larger to begin with. Scurvy symptoms were developed at the end of the third week and death supervened after 91 days.

No. 243 (initial weight 480 g.) did well, consuming an average of 85 cc. and increased in weight until the 25th day, when two living young were born. It immediately fell ill and died of a general septicaemia a few days later. No symptoms of scurvy were observed in life or at the post-mortem examination.

No. 235 (initial weight 260 g.) was started at the same time as, and in a manner in every way similar to, No. 234. From the first it co-operated well in the experiment and took its milk with enjoyment. It needed very little hand feeding. For three weeks it consumed an average of 84 cc. and put on weight steadily; later its daily average increased to about 120 cc., while on some days it consumed as much as 200 cc. It survived for more than 16 weeks; it more than doubled its weight, and during this period showed no symptom of scurvy.

The results of experiments in Groups I and II taken together suggest that it is the *quantity* of fresh milk consumed which is the important factor in influencing the survival of guinea-pigs upon a diet of oats, bran and fresh milk. If these results be compared with those summarised in Table I, where milk was omitted and where the guinea-pigs subsisted on oats, bran and water only, or with Table II where oats and bran and autoclaved milk were given, the following conclusions are reached with regard to the anti-scorbutic value of fresh milk:

1. When less than 50 cc. are taken daily, the animals showed no protection from scurvy.
2. When exactly 50 cc. are taken (No. 236) some degree of protection was apparent, life was prolonged, but the weight declined from a comparatively early date.
3. When 50–85 cc. are taken, increased protection from scurvy was noticed, and weight was better maintained (Nos. 232, 234, 242, 243).
4. In the one instance where 100–150 cc. was the daily ration (No. 235) the animal grew in a manner closely approximating to the normal (see Chart 4) and showed no symptoms of scurvy for 16 weeks.

Our interpretation of these results leads us to a concept of fresh cow's milk as a substance containing the anti-scorbutic accessory substance, but in small amount. It is therefore necessary that a large daily ration should be consumed, in fact that the diet should be composed entirely or almost entirely of milk, if scurvy is to be prevented by its agency alone. Such a concept is consistent with the rôle played in nature by mammalian milk, which is destined to form a complete food for a definite, but limited, period for the young of the species.

The low anti-scorbutic value of fresh milk is realised from Table II (*c*), where results obtained with more potent substances are given for purposes of comparison. It will be seen that scurvy in guinea-pigs can be effectively prevented by a daily ration of 2·5 to 5 g. fresh cabbage leaves, or 3 cc. fresh orange juice, whereas upwards of 100 cc. fresh cow's milk is required.

Our experimental work with fresh milk thus falls into line with other work pointing to scurvy as a "deficiency disease," caused by absence from, or deficiency in, the diet of an accessory food factor.

A similar set of experiments (already alluded to, p. 133), have recently been published by McCollum and Pitz [1917] and results resembling our own were obtained. The interpretation placed upon them by these authors was, however, entirely different from our own and led them to reject altogether the "vitamine hypothesis" of guinea-pig scurvy. They arrive at the conclusion that this disease is not due to any deficiency in the diet, but that it is the result of chronic constipation, caused by the physical structure of a "scurvy diet."

They are led to this conclusion by the fact that a certain diet, *i.e.*, rolled oats, casein, salts and butter fat (the last-named supplying the accessory growth factor, "Fat Soluble A") sufficient to maintain the rat in perfect health, is insufficient to maintain the guinea-pig. The latter goes down hill and dies, but whether from starvation and general inanition, or whether from the symptoms described by Holst and Frölich [1907, 1912] and other workers as scorbutic, *i.e.* haemorrhages, fragility of the bones and loosening of the teeth, together with specific histological changes in bone and bone marrow, is not stated. A further set of experiments with guinea-pigs is described, in which the diet consisted of rolled oats and fresh milk. In this case a certain proportion (two out of four) died of scurvy in 5–7 weeks; the remaining two animals survived, and it is argued that if some survive the diet must be chemically adequate for all. No allowance is made for individual idiosyncracy or for possible differences in the amounts consumed.

McCollum and Pitz consider that the metabolism of any two mammals, *e.g.*, the rat and the guinea-pig, must be similar, so that a diet which is chemically sufficient for the one must be chemically sufficient for the other, and if the one animal fails on it while the other survives, the failure must be due to some other cause than the deficiency in diet. They repudiate the idea that the guinea-pigs die from a "deficiency" scurvy and they attribute the deaths which occur on these diets to chronic constipation. They consider that the guinea-pig is peculiar in needing foods which will produce bulky easily eliminable faeces, and oats and milk they do not regard as belonging to such a category. They find support for this view in the fact that post-mortem examinations of guinea-pigs dead of scurvy generally show an empty stomach and a gorged caecum; further, they consider that scurvy in guinea-pigs can be both prevented and cured by the administration of laxatives, without any other alteration of the diet.

The results of experimental work carried out in this Institute [Chick and Hume, 1917] in confirmation of that of Holst and Frölich [1907, 1912, 1913], Fürst [1912], etc., in Christiania, have furnished abundant data in support of the explanation of guinea-pig scurvy as a deficiency disease, analogous as regards etiology, symptoms and methods of cure with the condition called scurvy in man. And, furthermore, the results which we have obtained by feeding guinea-pigs on common articles of human diet, including milk, correspond in a striking manner with current experience and old records of human scurvy.

We believe that the experiments of McCollum and Pitz will bear an entirely different interpretation from that which they have put upon them, an interpretation which falls into line with the "vitamine" hypothesis and requires no assumption of constipation as the causative agent. This interpretation we believe to be as follows: those guinea-pigs (2 out of 4) in their experiment which survived on a diet of unlimited fresh cow's milk and oats were those which consumed a large quantity of milk and very little oats, while those which developed scurvy were those that consumed much less milk. It is clear that an *ad libitum* ration of fresh milk may mean anything and as the quantities consumed were not measured, the above explanation could not emerge.

There remains the case of those animals developing scurvy on a diet of fresh milk and oats, whose condition improved after regular administration of a laxative [*e.g.* McCollum and Pitz, 1917, Nos. 2 and 4, Chart 7, p. 249]. A fall in weight coinciding with scurvy symptoms at about the 5th week, was checked and the animals did well subsequently. It will be found that weight charts, closely resembling these, are to be found where no curative doses were given [*e.g.*, McCollum and Pitz, 1917, Chart 3, p. 245, Chart 4, p. 246]. We have noticed that a distaste for solid food and greatly increased appetite for milk frequently accompanies an attack of scurvy. The improvement in weight following a fall, which was noticed in cases where a laxative was administered (and recorded also, without comment, in some cases where none was given) may possibly have corresponded to an increased consumption of the fresh milk. In our own experiments the length of life and welfare of the animals were found to be nicely graded to the amount of milk taken, and had McCollum and Pitz recorded the amount of milk actually consumed by their animals, we believe that their work would have yielded the same result.

As regards the constipation hypothesis of McCollum and Pitz, we have not observed that this condition is a constant feature of guinea-pig scurvy, indeed we should have said that diarrhoea with intestinal, duodenal and sometimes gastric haemorrhage, entered more often into the clinical picture. In some cases it is true, the failure to eat resulting from loosened teeth, does lead to a post-mortem picture showing an empty stomach and small intestine, and a stagnant caecum. No doubt cases of constipation occur in this way, but we have not found them the regular concomitant of scurvy symptoms. On the other hand many facts have emerged from the work in this Institute which are quite incompatible with the view that scurvy in guinea-pigs is caused by a chronic state of constipation.

Dr E. Marion Delf[1] has found that guinea-pigs can be protected from scurvy by 5 g. of peas, germinated for 48 hours [cf. Fürst 1912] when added to a diet of grain and milk (autoclaved at 120° for an hour). A very much larger ration, about 30 g., is needed to do this when the peas are merely soaked in water for 24 hours. It is hard to believe that the process of germination, which has proceeded for so short a time, can so alter the physical properties of a diet that its power of preventing or curing constipation should have increased sixfold.

McCollum and Pitz refer to Holst and Frölich's [1912] experiment with dried greens and suggest that the loss of power to prevent scurvy, which cabbage suffers on drying, is due to the failure of the greens to take up water in the intestine and to produce the bulky food needed to prevent constipation. We have repeated Holst and Frölich's work with cabbage, dried and kept for three months. Before being fed to guinea-pigs, the greens were soaked and became fully re-imbibed, yet an amount equal to more than 20 times the minimal ration of fresh cabbage was found incapable of preventing scurvy.

The changes in the food stuffs which are described in these two cases are not mere changes in physical condition but appear much more likely to be subtle chemical changes involving the appearance or disappearance of an, at present unisolated, chemical complex, such as a "vitamine" or accessory food factor is conceived to be.

It appeared worth while, however, to put the matter to an experimental test. Guinea-pig No. 278 (Chart 3 and Table III) received oats, bran and 50 cc. fresh milk daily; in addition 1 cc. liquid paraffin was administered every second day. This animal behaved like those which received a similar ration of fresh milk without any laxative. A fall in weight began on the 15th day, scurvy symptoms occurred on the 27th day.

In a second experiment guinea-pigs 269 and 270 were fed on a scurvy diet of oats, bran and autoclaved milk; 1 cc. liquid paraffin was given every second day, beginning from the 23rd day, the date at or about which scurvy symptoms were noticed. A few days later a steady fall in weight set in and death from scurvy took place on the 39th and 50th days respectively. The weight charts of these animals are set out in Chart 5, and their histories should be compared with those dealt with in Table II, where the animals received a similar diet without addition of any paraffin. The third curve in Chart 5 is concerned with guinea-pig No. 267, which developed scurvy on a diet of oats, bran and autoclaved milk, and was first treated with paraffin (as Nos. 269 and 270), but also received no benefit; later this treatment was changed for a daily dose of orange juice and immediate recovery from scurvy began.

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[1] Unpublished experiments.

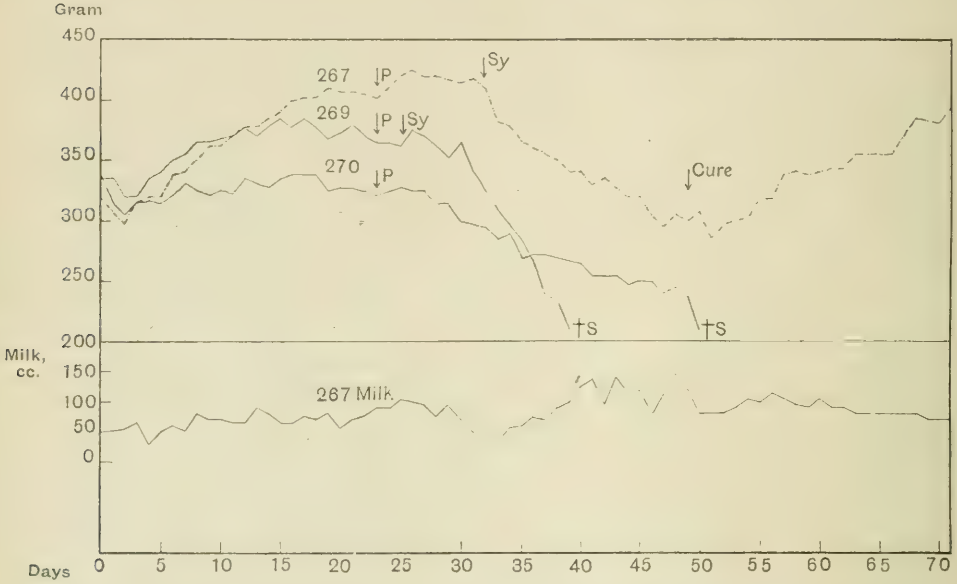

Chart 5. Weight curves of young guinea-pigs upon a diet of oats, bran and autoclaved milk.

↓ P signifies that 1 cc. liquid paraffin was given every second day subsequently.

↓ Sy signifies scurvy symptoms noted.

↓ cure signifies cure with orange juice started, paraffin discontinued.

†S signifies death from scurvy

Lower curve shows daily consumption of autoclaved milk in case of No. 267. It is interesting to note in this case that although the consumption of autoclaved milk increased from the 33rd day, the weight curve continued to fall steadily until after the orange juice was administered. Increased consumption of milk autoclaved at 120° C. for an hour had therefore no beneficial effect in scurvy.

Our experiments upon this point, as far as they go, do not, therefore, confirm the conclusion of McCollum and Pitz.

APPLICATION OF THE EXPERIMENTAL RESULTS TO THE QUESTION OF INFANT FEEDING.

The foregoing results indicate that anti-scorbutic substances are present in fresh cow's milk, but in small amount. It is therefore clear that when the milk is destined for the use of infants, any method of preparation which tends to diminish this small amount must be regarded with suspicion. Among these processes are included the heating and the drying of milk.

Use of Heated Milk.

It has become the almost universal custom to subject milk to preliminary heating before feeding to infants, in order to prevent transmission of bacterial disease.

Our own experiments with heated milk have only dealt with very severe heating indeed (at about 120° C. for one hour) and the ration taken has as a rule been less than 60 cc., an amount which is insufficient to protect against scurvy even when the milk is fresh. In the few cases, however, in which we have seen much larger amounts consumed, there was no sign that any anti-scorbutic substance was present which could assist in the process either of protection or of cure.

Frölich [1912] found that a diet of oats and milk, heated to 70° C. for 30 minutes or to 98° C. for 10 minutes, produced scurvy in guinea-pigs more frequently than did raw milk, but as he did not measure the quantities consumed, it is not possible to draw quantitative deductions from his work.

Practical experience of infant feeding also affords proof that damage is done when the heating takes place at temperatures lower than boiling point. Neumann [1902] states that in 1901–1902 he encountered 21 cases of infantile scurvy in his private practice, whereas previously he had rarely seen the disease. Of these 21 cases, 20 had for several months received milk from the same dairy, a dairy in which since 1901 it had been the custom to pasteurise the milk at 90–95° C. before delivery. In all cases the milk had been heated a second time after delivery in a Soxhlet or other apparatus, at or near 100° C., for 10–15 minutes. A similar experience is related by Heubner [1903]. This Berlin physician noted only 23 cases of infantile scurvy in Berlin between the years 1894 to 1900 inclusive. In 1901–1902 he met with 59 cases, of which 31 had been fed on milk previously heated in various ways and 11 had received condensed milk or patent food. He, in common with Neumann, attributed the marked increase in infantile scurvy at this period to the introduction of pasteurisation or boiling of milk before feeding to infants.

Perhaps the best instance in the literature is that studied in great detail by Hess and Fish [1914] and Hess [1916] in which 23 cases of mild infantile scurvy were shown to be caused by a diet for several months of cow's milk previously pasteurised at 145° F. (63° C.) for 30 minutes. This outbreak occurred at the Hebrew Infant Asylum, New York, where it had long been the custom to use pasteurised milk for the infants and in addition to give a regular ration of fresh orange juice. After the American Medical Milk Commissions had published the conclusion [1912] that, for purposes of infant feeding, heated milk might be considered as the equivalent of raw milk, the use of orange juice was discontinued in the Infant Asylum. The result was that an outbreak of mild scurvy took place about two to four months later. The babies were not acutely ill, and the symptoms were mild in comparison with the clinical picture of severe infantile scurvy. The patients were fretful, showed marked pallor, loss of appetite and some degree of anaemia; they also ceased to gain weight or to grow in length. In a few cases there were distinct haemorrhages in the mouth and elsewhere and in others a thin blue line of induration could be detected round the margin of the teeth. The infants were all more than six months old, and it would have been reasonable to attribute the illness to teething troubles. The scorbutic nature of the disorder was, however, proved by the fact that all symptoms cleared up speedily after restoring the extra anti-scorbutic to the diet or substituting raw milk for the heated milk.

Miller [1917] has described a series of similar cases from his private practice. The illness was mild, and other diagnoses were suggested, but successful cures following on treatment with anti-scorbutic material proved the trouble to be incipient infantile scurvy.

Use of Dried Milk.

Dried milk we believe to be largely, if not entirely, deprived of anti-scurvy vitamine. Whether this happens during the process of drying or in the long keeping which follows drying we are unable to say. We have, so far, discovered no conditions under which the anti-scorbutic vitamine can be preserved intact apart from living tissues for any considerable time at room temperature. It is slowly destroyed by some process at present unknown, and in dried milk there is no exception to the rule.

The history of two guinea-pigs (Nos. 163 and 162) are given in Table II, section (*b*). These animals consumed daily an average amount of dried milk equivalent to 60 cc. and 76 cc. milk respectively and there was no significant protection from scurvy. The loss of anti-scorbutic principle originally contained in the milk is seen by comparing the results of these two experiments with Experiments 2, 3 and 4 in Table III, where similar amounts of fresh raw milk were consumed.

If infants are nourished on dried milk or on "artificial" milk (prepared from dried skim milk with addition of vegetable fats) nutritive disorders are to be apprehended if the diet is maintained for several months, and if no extra anti-scorbutic is added. Expert medical opinion is divided upon this point, as upon the question of the adequacy of heated milk. Naish [1914], for example, believes that "the risk of scurvy upon such a diet is non-existent," and adds that he is not in the "habit of ordering any orange juice or other anti-scorbutic." Pritchard [1914, p. 304], on the other hand, states that "when dried milks or other preserved foods are employed, it is well to give some fresh fruit juice or other anti-scorbutic."

It must be remembered that scurvy is a disease which develops after a long period of imperfect nutrition, extending usually for several months. The symptoms will vary in severity according to the susceptibility of the individual and to the degree of deficiency in the diet. Every gradation may therefore be expected from almost imperceptible changes in mild cases to the well recognized severe symptoms of acute infantile scurvy. This point merits the consideration of those authorities who deny the need for extra anti-scorbutics in artificial feeding of infants on the ground that infantile scurvy is a very rare disease, in comparison with the great frequency of artificial feeding without extra anti-scorbutic. It is possible, however, that more careful observation may discover that in its milder manifestation, infantile scurvy is not so uncommon an ailment as has been suspected.

It is essential, where infants are concerned, that the very best conditions should prevail and it is the only prudent course, in the present uncertain state of our knowledge, to supply additional anti-scorbutic substance when anything other than fresh cow's milk is given. Of these, orange juice is easily the most suitable, but when the conditions are such as to put it out of the reach of the poor, raw swede juice may be substituted. We have found that when freshly squeezed, it is a valuable anti-scorbutic, though not as potent as orange juice; a larger dose should therefore be given.

Research at this Institute is now in progress to determine the exact relative values against scurvy of these and other foodstuffs, and also if possible to discover other anti-scorbutic materials which may usefully be employed in infant feeding.

SUMMARY.

(1) A set of nutritional experiments with young guinea-pigs is described in which the diet consisted of oats, wheat bran and fresh milk. The special feature of the work is the measurement of the amounts actually consumed.

(2) The basal scurvy-producing diet for control purposes consisted of oats, bran and water, or oats, bran, and 60 cc. milk autoclaved at 120° C. for an hour. With the former diet no growth was produced and it seemed possible that it lacked both the anti-scorbutic factor and the "Fat Soluble A" growth factor of McCollum and his co-workers. With the addition of the autoclaved milk growth took place until the onset of scurvy symptoms; it was concluded therefore that this diet was in every way a sufficient one, save only that it lacked the anti-scorbutic factor.

(3) If the daily consumption of fresh milk was less than 50 cc. the guinea-pig developed scurvy in a period not different from that in the control experiments.

(4) If the daily ration varied from 50–100 cc., a greater or less protection from scurvy was observed, varying proportionately with the amount consumed.

(5) If 100–150 cc. was taken daily, satisfactory growth and development occurred and no symptoms of scurvy were observed. This practically amounts to a complete milk diet.

(6) The above facts accord well with the vitamine deficiency hypothesis of the etiology of guinea-pig scurvy. Milk is evidently a food poor in the anti-scurvy accessory factor and a ration large in comparison with that of other anti-scorbutic materials is necessary to afford satisfactory protection from scurvy.

(7) In our opinion these experiments offer a reasonable explanation of the anomalous results obtained by other observers, when guinea-pigs were fed on diets consisting of grain and fresh milk, and when no measurements were made of the amount of milk actually consumed. Irregularity was noticed in the onset of scurvy symptoms and the observers were led to seek an explanation of the ill-health in causes other than a deficiency in diet. Jackson and Moore [1916] and Jackson and Moody [1916] have arrived at the conclusion that guinea-pig scurvy is an infection of bacterial origin; McCollum and Pitz [1917] think that it is due to chronic constipation, caused by the physical properties of the oats and milk diet.

(8) We have obtained no evidence that chronic constipation is a constant concomitant of guinea-pig scurvy. Instances are given, where certain

modifications in diet, to which no extra laxative effect can be attributed, have cured or prevented guinea-pig scurvy. Experiments are also described in which the administration of a laxative alone has failed to cure or prevent scurvy. The results, on the one hand, afford additional confirmation to the theory that guinea-pig scurvy is due to the deficiency in the diet of a specific accessory substance and on the other hand to the theory that it is analogous in etiology and method of cure with human scurvy.

(9) Application of the results is made to infant feeding with cow's milk and attention is called to the risk of tampering with a substance which even in the fresh condition is but feebly anti-scorbutic. In order to give the child optimum conditions, it is strongly urged that whenever milk is heated in any way, or dried, an additional source of anti-scorbutic vitamine should be provided. Clinical experience and animal experiments point to orange juice as the most suitable anti-scorbutic to employ, but if, owing to war or other conditions, it is unobtainable, raw swede juice in rather larger doses may be substituted.

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XII. THE EXAMINATION OF THE FAECES OF RABBITS FED ON A DIET OF CABBAGE FOR THE OCCURRENCE OF A PHYTOSTEROL, INCLUDING A NOTE ON THE PHYTOSTEROL IN CABBAGE SEEDS AND THAT IN GRASS FRUITS.

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Previous experiments by Dorée and Gardner [1908] on the excretion of cholesterol in the animal organism, led to the belief that the phytosterols and similar bodies occurring in the plant given as food appear in the faeces in an unchanged form.

The examination of the product when grass was given as food has already been carried out by these authors. The chief body in the faeces under these conditions is chortosterol, an alcohol having the formula $C_{27}H_{54}O$, or $C_{27}H_{56}O$, which does not give the cholesterol colour reactions, and this substance was also found in grass leaves. Phytosterol itself was either absent, or present in the oily matter of the unsaponifiable ether extract in too small amount to separate. When bran was given in the food, the phytosterol of bran was found in the faeces. Further, from results obtained by Fraser and Gardner [1909] it appeared that when phytosterol was given in the food of rabbits, the greater part appeared in the faeces. Further, experiments carried out later [Fraser and Gardner 1910] by the same authors showed that although phytosterol given in the food increased the cholesterol content of the blood, it did not appear in the blood as such, and must have been altered in form or excreted. In continuation of this work, an examination of the faeces of rabbits fed on a diet of cabbage was made by the author, to determine whether a phytosterol was present, and to investigate the change, if any, in this substance in its passage through the digestive organs.

Rabbits in the laboratory were fed on cabbages, some on the outer leaves and others on the inner stem, including the apical bud—40 kilos of leaves, 30 kilos of stalk were used. Their faeces were collected and extracted separately. The account will thus be divided into results obtained with leaves and with stalks.

The faeces were dried, and then ground up as finely as possible. The dried material was subjected to prolonged extraction with ether in a Soxhlet apparatus. The ether extract thus obtained was saponified with a large excess of alcoholic sodium ethylate according to the method of Kossel and Obermüller. In order to insure complete saponification the saponified liquid was always allowed to stand over-night. The precipitate of soap was separated by filtration, and washed with ether to remove all the ether-soluble substances. The filtrate was shaken up with water several times to free it from excess of alkali. Finally the washed ether extract was evaporated to dryness, the residue dried and kept for examination.

The Leaves.

On saponification it was observed that very little soap was present. The dried ether extract consisted of solid matter, not obviously crystalline, in a brown oil. This matter proved exceedingly difficult to separate, as the brown oil came out of solution with the solid from ethyl alcohol, acetone, and ethyl acetate. The solution in acetone was therefore boiled with charcoal, and afterwards filtered hot. The charcoal was subsequently extracted several times with boiling acetone to remove the substances as completely as possible. From this solution a pure white substance was obtained in small crystals; in granular lumps from acetone and ethyl acetate. It was finally recrystallised from ether, from which it separates in beautiful rosettes of lustrous crystals.

(1) The first substance to come out of solution in ether melts at 78°–79°. It separates from alcohol as a jelly, in a more granular form from acetone and ethyl acetate; from ether and chloroform in much larger rosettes of crystals. It does not give the cholesterol reactions and does not form an acetate. The similarity in properties made it probable that it might be chortosterol, but as the acetate could not be obtained this could not be proved. The amount of the substance was too small for further investigation, but it was evidently not a phytosterol.

(2) The mother liquors deposited a crystalline solid melting between 60°–75°; this was evidently a mixture and proved very difficult to separate.

Finally a substance of M.P. 63°–64° was isolated which gave a constant melting point after several recrystallisations. It is very light, and separates in a crystalline form from ethyl acetate, acetone, ether and chloroform. From acetone, when the substance is pure, the crystals under the microscope show the form of 4-sided plates. Salkowski's and Liebermann's tests are negative. It has no specific rotation.

From its properties it evidently does not belong to the sterol group, and is probably one of the hydrocarbons or alcohols occurring in plant waxes.

(3) The mother liquors consist of a heavy brown oil, containing a trace of crystalline matter, too small for identification. This gives the cholesterol colour reactions.

The Stalks.

The unsaponifiable residue from the faeces of rabbits fed on cabbage stalks was very similar to that from the leaves in appearance. The solid crystallising out was difficult to separate from the brown oil present, and the residue was dissolved in acetone and boiled up with charcoal.

Two substances were isolated.

(1) The less easily soluble substance which came out of solution almost completely in the cold from most solvents melted between 60° and 70°. After repeated crystallisation from ether, a substance melting at 63°–64° was separated in small amount. It had the same crystalline properties as that from the leaves, and the M.P. was not altered by mixing the substances from the stalks and leaves in equal proportions.

(2) The more soluble substance remained behind in the mother liquors. On evaporation to small bulk a distinctly crystalline body separated out from the oily solution in rosettes of long needles. The substance was finally purified from absolute alcohol. The melting point was 102°, and the substance gave the cholesterol colour reactions. Only a very small amount of this substance was present, so that it was difficult to identify it further.

THE TISSUES OF THE CABBAGE.

An examination of the dried leaves and stalks of the cabbage itself showed that the unsaponifiable ether extract was very small in amount. It consisted mainly of the same substances as appeared in the faeces, and although the mother liquors gave the phytosterol colour reactions, no crystalline substance like phytosterol was isolated. It appeared from these results that a typical phytosterol, if present, only occurred in the cabbage plant to a very small extent, and as a comparative experiment, the seeds were examined to find out if they contained this substance.

The Seeds. Two kilos. of cabbage seeds were ground up very finely and extracted with ether. The extract was saponified in the usual way, and the unsaponifiable matter, after drying, weighed 14·9274 g.

It consisted of a mixture of crystalline matter with a brown oil. The residue was recrystallised from alcohol, and finally purified from solution in acetone. The crystals from alcohol were glistening plates, from acetone smaller. They gave the cholesterol colour reactions; the melting point of the pure substance was 142°. A substance with the same melting point was described by Bömer [1899, 1901] and later by Windaus and Welsch [1909] in rape oil. The latter authors were able to show that it was a mixture of two substances: (1) brassicasterol, $C_{28}H_{46}O$, M.P. 148°, and (2) a phytosterol $C_{27}H_{46}O$, M.P. 142°. They obtained the bromide of the acetate, and found that brassicasterol formed a tetrabromide insoluble in glacial acetic acid, whereas the other substance formed a dibromide which remained in solution.

This phytosterol resembles the sitosterol described by Burian [1897] and later by Ritter [1902] in wheat and rye embryos.

In order to determine if the substance isolated from cabbage seeds was the same as that in rape oil the following experiments were carried out.

Preparation of the acetate. 0·05 g. of the substance was mixed with 0·1 g. of sodium acetate, and a few cc. of acetic anhydride added. The mixture was boiled under a reflux condenser for ¾ hour and then poured into much water and allowed to stand over-night. The precipitate was then filtered, washed free from acetic acid, and dissolved in alcohol, from which it was twice recrystallised. The crystals melted at 137°. This seemed so similar to the mixtures of substances in rape oil, that it was interesting to observe if a tetrabromide would be formed.

Preparation of the bromide of the acetate. A further preparation of the acetate was carried out, and about 0·25 g. was dissolved in 2 cc. of ether, and 3 cc. of a 5 % solution of bromine in glacial acetic acid were added. After some time crystals separated out. To determine if these were identical with those of the tetrabromide of the acetate of brassicasterol, they were purified by recrystallisation from absolute alcohol, but the results were not very conclusive. The tetrabromide of the acetate of brassicasterol described by Windaus and Welsch, melts at 209°. The melting point of the substance just described was lower. It is hoped later to carry out the experiments on the same scale as was done by Windaus and Welsch, who used much larger quantities.

The results obtained from these experiments: that the cabbage seeds contain a relatively large amount of a mixture of phytosterols, whereas the vegetative organs seem to contain a very small amount, made it interesting to compare the investigation of grass described by Dorée and Gardner [1908] with an examination of the fruit.

GRASS FRUITS.

515 g. of grass fruits were extracted, giving an unsaponifiable residue weighing 2·05 g. From this residue on crystallisation from acetone, the first substance which separates out in small quantity, resembles chortosterol in its solubility, but it was too small in amount to be purified satisfactorily. It is probably derived from the leaf-structures surrounding the grass fruit, and inseparable from it. From the mother liquors, 0·07 g. of a crystalline substance resembling phytosterol was obtained. This melted at 135°–137° and gave the cholesterol colour reactions.

0·04 g. was converted into the acetate, which after recrystallisation melted at 119°–120°. This remained unaltered on subsequent recrystallisation.

It would seem, from the data available, that this is a mixture of sitosterol and some similar body, but the quantity was too small to attempt separation of the constituents.

SUMMARY AND CONCLUSIONS.

(1) The chief constituent of the unsaponifiable ether extract of the faeces of rabbits fed on a diet of cabbage, was not phytosterol, but a mixture of substances of lower melting point, probably hydrocarbons or alcohols such as are known to occur in plant waxes. A typical phytosterol was not isolated either from the faeces or from the vegetative organs of the cabbage plant itself, although a small amount of a substance melting at 102° and giving the cholesterol colour reactions was separated from the faeces of rabbits fed on cabbage stalks. This might be coprosterol, although that substance has never been found in the faeces of the herbivora, but the amount was too small for complete identification.

(2) Cabbage seeds, on the other hand, contain relatively a large amount of crystalline matter, which seemed similar to the mixture of phytosterols present in rape oil. This is interesting as rape and cabbage are different species of the same genus—*Brassica.*

(3) Grass fruits also contain phytosterol, although the chief constituent of the unsaponifiable ether extract of the leaves is chortosterol, which does not give the cholesterol colour reactions.

(4) These results seem to indicate that whereas phytosterol is present in the seeds, in the vegetative organs it is absent or present in relatively small amount.

Such a conclusion, however, would hardly be justified unless some more accurate method of separation were employed, permitting of the separation of phytosterol which may be present in the oily residues, as they give the phytosterol colour reactions.

Further experiments are in progress, and the details will be given in a later communication.

In conclusion the author would like to express her thanks to Professor Waller and to Mr Gardner for the facilities afforded in the Physiological Laboratory.

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XIII. CONTRIBUTIONS TO OUR KNOWLEDGE OF THE PLANT STEROLS. PART I. THE STEROL CONTENT OF WHEAT (*TRITICUM SATIVUM*).

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In accounts of the chemical analysis of plants, "a phytosterol" is often mentioned among the constituents. Particularly noteworthy in this connection are the researches of Power and his co-workers at the Wellcome Chemical Research Laboratories.

Descriptions of the constituents of plants or parts of plants of pharmaceutical importance have appeared during recent years in the *Journal of the Chemical Society*—and in pharmaceutical journals—and it would seem from these that phytosterol is present in seeds, roots, leaves, shoots, flowers—in latex and in bark.

In addition to this the phytosterols from special sources have been examined very carefully by various authors. The substances so described are isomeric with the animal cholesterol, in most cases having the formula $C_{27}H_{46}O$, but included in the group are certain other compounds possessing slightly different formulae which may be regarded as very nearly related. Chemically, like cholesterol, they react as unsaturated alcohols forming bromine additive compounds, and esters. They are generally optically active, and they give the cholesterol colour reactions—though sometimes in a modified form. The two chief tests of this kind are:

(1) *The Salkowski Test*—in which a few mg. of the substance are dissolved in about 2 cc. of chloroform and shaken with an equal bulk of strong sulphuric acid. The chloroform develops a blood-red or cherry-red colour which it retains for several days. On pouring this solution into a basin, the colour changes to blue, green, and then yellow, owing to absorption of water. On addition of sulphuric acid, the original colour is restored. The sulphuric acid layer is distinctly fluorescent.

(2) *The Burchardt-Liebermann Test.* A few mg. of the substance are dissolved in 2 cc. of chloroform, 20 drops of acetic anhydride added, and one drop of concentrated sulphuric acid. A violet pink colour appears. This test will detect very small traces of sterol, but it is also given by resins.

Phytosterols usually have a characteristic crystalline form. From hot alcoholic solution, bundles of glistening crystals appear on cooling, and, when slowly crystallised, these are found to be well-defined hexagonal plates.

Whereas cholesterol itself is found to be a constant constituent of animal protoplasm, in the vegetable kingdom it seems that a number of isomers exist, differing from one another to a slight extent in their properties, notably in their melting points and the melting points of their esters. These substances form mixtures which crystallise as if only a single substance were present. Mixtures of the plant sterols with cholesterol (which has not so far been found in plants) behave in the same way. Many of the substances described melt between 130° and 138°, but compounds with higher melting points have been described.

The differences in these substances are not much greater, however, than those observed in the case of the five dihydrocholesterols which have been described and which can easily be changed one into the other, as has been shown by Dorée and Gardner [1908, 1] in the case of coprosterol and ψ-coprosterol and in the case of the rest by Windaus and his co-workers [Windaus and Uibrig, 1914, 1915].

According to the researches of Windaus [1916] these bodies possess in a high degree the capacity of crystallising together so as to simulate a single compound. The various isomers in Windaus' opinion differ only in the stereoposition of H and OH groups with regard to an asymmetric carbon atom.

These varying plant sterols, as already indicated, are very widely distributed. The best known perhaps are those occurring in:

(1) *Seeds.* Hesse [1878] gave the name "phytosterol" to a substance present in Calabar beans, to which he assigned the formula $C_{26}H_{44}O + H_2O$. It melted at 132–133° and formed an acetate, M.P. 120°.

A similar substance had been described by earlier writers, Beneke [1862] in seed peas, Ritthausen [1863] in wheat gluten, Lindenmeyer [1863] in peas, but these authors considered it to be cholesterol. After that, a number of papers appeared describing phytosterols melting between 132° and 137°.

The formulae assigned often differ slightly from $C_{27}H_{46}O$, but the method of combustion is not entirely conclusive unless many determinations of the substance and as many esters as possible are made, since the percentage carbon and hydrogen content would differ but little in compounds of such high molecular weight.

The phytosterols described are isolated in very small quantities, and one needs a relatively large quantity for satisfactory investigation.

Jacobson [1888] described the phytosterol of peas and beans, and in 1897 a great advance was made by Burian [1897] who isolated from wheat and rye embryos a substance which he named sitosterol, and considered to be isomeric with cholesterol. This melts at 137·5°. It is soluble in most organic solvents, crystallises from 90 % alcohol with one molecule of water of crystallisation and in ether has $[\alpha]_D = -26°·71$.

He prepared many of the derivatives of sitosterol and characterised the substance fairly completely. The acetate melted at 127° and the benzoate at 145–145·5°. Cholesterol benzoate shows a fine play of colour on cooling, and sitosteryl benzoate does this in a less pronounced fashion.

Ritter [1902] confirmed this work, and added to our knowledge of the phytosterol from wheat germ. After sitosterol had been thus characterised, it seemed probable that the phytosterols described as melting between 132° and 137° consisted of this substance with varying amounts of some similar compound as impurity.

Owing to the resemblance in their properties the separation of these mixtures presented unusual difficulty.

Windaus and Hauth [1907], however, were able to show that the original phytosterol of Hesse, from Calabar beans, was really a mixture of sitosterol, identical with that in wheat germ, and another alcohol, stigmasterol, M.P. 170°, having the formula $C_{30}H_{48}O$. This latter compound has two double linkages in the molecule and the acetate forms a tetrabromide. This tetrabromide settles out from glacial acetic acid on standing while the sitosteryl acetate dibromide remains in solution.

(2) *In Oils.* As already mentioned, phytosterol has been described as a constituent of many plant oils, notably by Bömer [1899] in cotton-seed oil, ground-nut oil, sesamé oil, colza oil, poppy oil, hemp-seed oil, linseed oil and castor oil. He finds that the M.P. differs slightly according to the source, but the elementary composition is the same. Sani [1903] finds a phytosterol in olive oil, and rape oil was examined first by Bömer [1899, 1901] and later by Windaus and Welsch [1909]. The latter authors showed it to be a mixture, and, by means of the bromide of the acetate, isolated two separate sterols—one melting at 148° which they named brassicasterol. This had the formula $C_{28}H_{46}O$, and like stigmasterol formed a tetrabromide of the acetate. The

other had a lower melting point, 142°, and the authors found that this was a phytosterol with the formula $C_{27}H_{46}O$.

Cocoa butter was found to contain a mixture of two phytosterols by Matthes and Rohdich [1908, 1, 2] but Heiduschka and Gloth [1908] consider that cotton seed oil contains a single substance as no tetrabromide is formed.

The capacity of mixtures of cholesterol and the plant sterols to crystallise as a single substance, is made use of in the detection of adulteration of animal oils by those of plant origin. The characteristic four-sided tablet of cholesterol is always altered by an admixture of phytosterol.

(3) *In Roots and Rhizomes.* Perhaps the earliest mention of the substance occurs in the analysis of carrots by Husemann [1861] under the name of hydrocarotol. This was later described by Euler and Nordensen [1908] under the name of daucasterol. Tutin and Clewer [1912] describe a phytosterol verosterol, from rhubarb rhizome, and Power and Salway [1914, 1] from sarsaparilla root. Rumpler [1903] gives the name betasterin to a phytosterol of M.P. 117° from beetroot.

(4) *In Stem, Leaves and Flowers.* Phytosterols have been found in the flowers of plants, in those of *Anthemis nobilis* for example, as anthesterol, by Klobb [1902] and also by Power and Browning [1914], in the flowering branches of *Clematis vitalba* by Tutin and Clewer [1914] and in the leaves and stems of *Daviesia latifolia* by Power and Salway [1914, 2]. Certain substances analogous to phytosterol have been found in olive leaves by Power and Tutin [1908]: oleasterol, $C_{20}H_{33}OH$, M.P. 174°, olestranol, $C_{25}H_{42}O_2$, M.P. 217–218° and homo-olestranol, M.P. 210°.

(5) *In Latex.* Cohen [1908] has obtained mixtures of phytosterols from South African rubber, and various other bodies from latex have been described.

In addition to the isolation of phytosterol itself as a constituent of plants, Power and his co-workers made the very important discovery that it was present as a glucoside. In their analyses of plants there often occurred high-melting compounds, which seemed to be alcoholic in nature, with more than one hydroxyl in the molecule. These gave the cholesterol colour reactions, but the M.P. and the formulae served to differentiate them from true phytosterols.

As an example, ipuranol, $C_{23}H_{38}O_2(OH)_2$, M.P. 285–290°, occurred in the stems of *Ipomoea purpurea*, in olive bark and in nutmeg. In taraxacum root, cluytianol was found, bryonol in bryony root, and similar substances in various plant organs. A list is given in the paper by Power and Salway [1913] which describes their identification as phytosterol glucosides. They were unchanged by the usual methods of hydrolysis, but when heated with aqueous hydrochloric acid in amyl alcohol solution, dextrose and a phytosterol were formed. Different phytosterols were obtained from different glucosides and it is probable that different sugars may form part of the molecule.

These phytosterol glucosides are named phytosterolins. They may occur in the plant as well as phytosterol, but in the leaves of *Prunus serotina*, according to Power and Moore [1910], phytosterol is not present except in combination as a glucoside.

The foregoing evidence of the occurrence of a phytosterol in so many different plants and different organs of plants, suggests that in the higher plants, at least, it is a constant constituent. In the lower plants comparatively few phytosterols have been isolated, and these are described chiefly in the fungi.

The best-known representative is the ergosterol of Tanret [1889]. This was first isolated from ergot, melted at 154°, and gave an acetate melting at 159–175°. Later it was further examined by the same author [Tanret, 1908] and shown to be a mixture of two substances: (1) ergosterol, $C_{27}H_{42}O$, melting at 165°, forming an acetate melting at 185°, and possessing an unusually high specific rotation; and (2) fongisterol, the lower homologue, $C_{25}H_{40}O$, M.P. 144°; this is more soluble in the different solvents, has a much lower specific rotation, and gives an acetate melting at 158·5°. These substances are further distinguished by a colour test. Ergosterol gives no colour with strong sulphuric acid at the end of one minute, while fongisterol becomes coloured ruby-red after some seconds, changing to red-violet later.

Gerard [1892, 1895] found an ergosterol present in *Penicillium glaucum*, and *Aethalium septicum*, in *Mucor mucedo*, and in *Lobaria pulmonacea*, and he divides the plant sterols into two groups: (1) the phytosterols, (2) the ergosterols. The first group occur in the higher plants, and usually have lower melting points. The second group occurs in the lower plants, and have higher melting points. The two groups are distinguished by their colour reactions. Zellner [1905, 1908, 1, 2, 1911] describes similar bodies in fat of ergot, in *Amanita muscaria* (the fly agaric), in *Trametes suaveolens* and in *Polyporus ignarius*.

A phytosterol is described in all seeds examined, and in vegetable oils derived from seeds and fruits, but no experiments have hitherto been carried out to trace the fate of this phytosterol in the subsequent development of the plant. It has been described as occurring in all the different organs, but it seemed important to ascertain whether its occurrence was limited to any special organ, and further if it were possible from such occurrence to form any conclusion as to its probable function.

The results of the examination of the faeces of rabbits fed on a diet of cabbage, of cabbage leaves and stems, and of cabbage seeds [Ellis, 1918], led to the conclusion that while phytosterol was present in relatively large amounts in the seed, the quantity in the vegetative part was small. It seemed necessary to test this conclusion by some more systematic method of experiment, and if possible by the employment of accurate quantitative means. The brown oils giving the cholesterol colour reactions may contain appreciable amounts of a crystalline phytosterol, although it is very difficult to isolate it by the ordinary methods of solution and crystallisation.

For this purpose the wheat plant was chosen partly because the sitosterol from the germ has been so fully characterised by Burian [1897] and later by Ritter [1902], and partly because this sitosterol forms a compound with digitonin to be referred to later. Its economic importance also gives experiments on wheat a value from the utilitarian point of view.

The following experiments were carried out:

(1) The amount of phytosterol present in the whole grain was estimated, and this substance was characterised. Bran and wheat germ meal were also examined, the two latter representing the seed-coat and the germ respectively as nearly as it is possible to obtain them on a large scale.

(2) A known weight of grain was planted and the resulting wheat allowed to grow under normal conditions until well developed. The plants were then carefully collected and the phytosterol content examined.

(3) In addition to the normally developed plants, experiments were carried out with etiolated plants.

Leaves and roots were examined separately in certain of the experiments.

In every case the unsaponifiable ether extract, which would contain any sterol present, was obtained in the following way. The plant material was dried, and then ground up very finely in order to render all the tissues permeable. The dried material was then subjected to prolonged extraction with ether in a Soxhlet apparatus. The ether extract so obtained was saponified with a large excess of alcoholic sodium ethylate, according to the method of Kossel and Obermüller. In order to insure complete saponification the saponified liquid was always allowed to stand over-night. The precipitate of soap was separated by filtration, and washed with ether to remove all the ether-soluble substances. The filtrate was then shaken up with water several times to free it from excess of alkali. Finally the washed ether extract was evaporated to dryness, the residue dried and examined.

These experiments and their results are given in detail below.

The Grain.

3·05 kilos. of grain were ground up very finely and extracted with ether. The ethereal extract was saponified, washed, and the ether evaporated to dryness. The residue consisted of crystalline matter mixed with oil. After drying it weighed 1·528 g. From this about 0·95 g. of crystalline substance was obtained, giving a yield of approximately 0·031 %.

The pure crystals resemble phytosterol and give a melting point of 137·5°. They give the cholesterol colour reactions. This appears to be the same body as that described by Burian and Ritter in the embryo. To identify it further the acetate was prepared in the way previously described. After recrystallisation from acetone, the dried crystals melted at 127°, showing that the chief phytosterol present in wheat grain is that of the embryo—sitosterol.

The Bran.

1·25 kilos. of bran were extracted, saponified, and the resulting ether extract evaporated to dryness. The residue weighed 1·267 g. It was repeatedly recrystallised from 95 % alcohol, and finally a substance was obtained melting at 142° which gave the cholesterol colour reactions and resembled phytosterol in crystalline form. This was converted into the acetate by heating with acetic anhydride and sodium acetate, and the resulting compound was recrystallised. It melted at 137°. The benzoate was prepared by dissolving the substance in dry pyridine and adding benzoyl chloride until a faint yellow colour appeared. After standing over-night this was poured into much water and filtered. The precipitate was dissolved in hot alcohol and recrystallised from that solvent. Under the microscope the crystals appeared as hexagonal plates. They melt at 133-134°. The esters on saponification yield the original substance, melting at 142°.

This phytosterol differs from sitosterol in the melting point of its esters, but for its further characterisation much more material would be necessary than was available.

The Wheat Germ Meal.

1·8 kilos. of meal were extracted. The unsaponifiable residue consisted almost entirely of crystalline matter and weighed 10·522 g. This gives a yield of about 0·5 %.

The Normal Wheat Plants.

500 g. of autumn wheat were planted in November, and allowed to grow until May of the following year. These plants were grown at the Chelsea Physic Garden, and the author takes this opportunity of thanking the Curator, Mr Hales, for his very kind help in superintending the plants. The plants were collected as carefully as possible and shoots and roots dried separately. The total dry weight of plants was 2250 g. When dry they were well ground up and extracted with ether.

Shoots and roots will now be described separately.

The Shoots. The dry shoots weighed 1950 g. This material was well ground up and extracted with ether. The weight of ether extract obtained was 125·69 g. It was very dark green, greasy looking, and had a curious strong odour. 48 g. of this was taken for saponification. The unsaponifiable residue weighed 5·489 g. The residue was dark orange colour, oily, with characteristic odour. It consisted of a solid mixed with a brown oil, but was not obviously crystalline. This residue was dissolved in alcohol, from which it separates in a jelly-like form, very difficult to filter. When ethyl acetate is employed as a solvent, the substance comes out in more granular form.

It was recrystallised from this solvent, but it was only obtained pure after repeated recrystallisation. The first crop of substance melted at 80°, after recrystallising at 83°. It was so difficult to separate further quantities of this solid from the oil, that a second portion, 37·73 g., of the ether extract was saponified. The unsaponifiable extract, which weighed 4·4772 g., was dissolved in ethyl acetate and boiled up with about an equal quantity of charcoal, the charcoal being subsequently repeatedly extracted with boiling ethyl acetate.

Alcohol ($C_{20}H_{42}O$). The ethyl acetate solution yielded a pure white substance after several recrystallisations. It separates from ethyl acetate and acetone in granular lumps of small crystals. The pure substance melts at 83°. The elementary composition corresponds most nearly to $C_{20}H_{42}O$.

0·1024 g.; 0·3016 g. CO_2; 0·13365 g. H_2O.

Calculated for $C_{20}H_{42}O$	Found
C = 80·54	C = 80·32
H = 14·09	H = 14·50.

It was converted into the acetate in the usual manner, and the resulting substance purified by recrystallisation from acetone. The acetate was very difficult to filter on account of its soft consistency. It melted at 65°. The substance did not give the cholesterol colour reactions, and was not a phytosterol. Etard [1892] describes an alcohol, medicagol, from lucerne leaves to which he assigns the formula $C_{20}H_{42}O$. He says it forms a benzoate, but does not give figures. An interesting point about his paper is that he mentions experiments, the results of which showed that when a horse was fed with lucerne leaves, the alcohol was unaltered by passage through the digestive tract of the animal, and appeared in the faeces. This agrees with the conclusions arrived at by Dorée and Gardner [1908, 2].

Haller [1907] describes an alcohol occurring in the wax from *Raphia ruffia*, M.P. 80°, to which the formula $C_{20}H_{42}O$ is assigned, but no figures are given. A mixed melting point with the medicagol of Etard (from whom he obtained a specimen) shows that they are not the same. The acetate described by Haller seems to be the same as that obtained by the author, M.P. 65°, who concludes that the alcohol occurring in wheat leaves is the same as that in *Raphia ruffia*. It is not identical with arachyl alcohol, which melts at 71°. As it was not a phytosterol, the investigation of this substance was not carried further. The mother liquors deposited crystalline matter resembling phytosterol, but the small amount present was difficult to separate from the brown oil accompanying it in order to weigh it directly.

A method of estimation by means of the compound with digitonin was carried out. Windaus [1909] discovered that cholesterol, but not cholesterol esters, combines quantitatively with digitonin to form a highly insoluble compound, digitonin cholesteride, according to the following equation:

$$C_{55}H_{94}O_{28} + C_{27}H_{46}O = C_{82}H_{140}O_{29}.$$

This compound is insoluble in water, acetone, ethyl acetate, ether and benzene.

The residue containing the sterol is dissolved by boiling in 95 % alcohol, and to this solution is added excess of digitonin in 95 % alcohol. The mixture is allowed to stand for some time, then evaporated to dryness. The precipitate is washed by decantation with ether into a previously weighed Gooch crucible until the ethereal washings give no residue on evaporation. After that it is boiled up with water, transferred to the crucible and washed with hot water until the washings no longer froth on being shaken. The compound is somewhat hygroscopic, and the crucible and contents are therefore dried for some time at 110° and weighed in a stoppered vessel, the drying being continued until the weight is constant. This method has been worked out by Gardner and the author [Fraser and Gardner, 1910] in order to estimate small amounts of cholesterol in animal tissues. The amount of digitonin available was not sufficient to allow of the method in its application to the plant sterols being worked out completely.

It is hoped that the author may carry out that investigation at some future period.

For the purpose of this research, however, use was made of the fact that Windaus found that sitosterol and other well-known isomers of cholesterol formed analogous compounds with digitonin, with similar solubilities. A preliminary investigation with the pure substance, sitosterol, was carried out, and it was found that 0·001 g. sitosterol could be accurately measured by this means. In the case of wheat stalks the alcohol $C_{20}H_{12}O$ was removed by filtration, and washed with acetone until it no longer gave the cholesterol colour reactions. The filtrate and washings were then evaporated to dryness, dried and weighed. The weight was 1·752 g. As the amount of digitonin was limited and there was not enough to precipitate the whole of this residue and those of the other material to be presently described, the following procedure was adopted. The residue was dissolved in 20 cc. of 95 % alcohol, the solution well mixed and 2 cc. removed for the estimation, this forming a tenth part of the whole residue. The weight of compound obtained was 0·1087 g. equivalent to 0·2639 g. phytosterol from the residue from 37·73 g. This gives a total weight of 0·8802 g. phytosterol from 125·69 g., the weight of the ether extract of the shoots. On a dry weight of 1950 g. the percentage content of phytosterol is thus 0·045.

The Roots. The dried wheat roots from the normal plants weighed 300 g. The ether extract weighed 1·451 g. It consisted of a small amount of a solid apparently the same as that in the shoots and a brown oil. There was some appearance of crystalline matter. Exactly the same procedure was carried out as for the estimation of phytosterol in the shoots. The residue gave 0·113 g. of sterol, or a percentage of 0·037 on the dry weight of the roots.

ETIOLATED WHEAT PLANTS (1).

500 g. grain were planted at the Chelsea Physic Garden in November. They were covered with canvas bags to exclude the light. A few of the plants showed green in spite of this precaution, but in March signs of withering began. The plants were then carefully collected and dried, shoots and roots being separated.

The total dry weight of the plants was 203·5 g.

The Shoots. 203·5 g. dried plants gave 135·2 g. dry weight of shoots. This was extracted with ether, and saponified as before. The unsaponifiable ether extract weighed 2·175 g. It consisted chiefly of the alcohol $C_{20}H_{42}O$ present in the normal shoots, melting at 83°, recrystallised from acetone.

From solution in acetone the mother liquors deposited a distinctly crystalline residue. The procedure adopted for the normal shoots was carried out. The amount of phytosterol present in the residue was 0·0674 g. a percentage of 0·0487 on the dry weight of shoots.

The Roots. The dry weight of the roots was 68·5 g. The unsaponifiable ether extract weighed 0·5702 g. This gave 0·0855 g. phytosterol, a percentage of 0·1252 on the dry weight of roots.

ETIOLATED WHEAT PLANTS (2).

383 g. wheat grain were grown on filter paper in an incubator in the laboratory. The plants, when grown, were completely etiolated. The dried material weighed 168 g. The unsaponifiable extract from this weighed 1·404 g. The amount of phytosterol present was 0·1175 g. or 0·07 % of the dry weight of plants.

These results are put together in the following table:

Table showing Sterol Content of Wheat grown under varying conditions.

Experiment		Weight of grain planted in g.	Dry weight of material in g.	Total dry weight of plants in g.	Amount of phytosterol in g.	Total amount of phytosterol in plants in g.	Percentage of phytosterol in roots and shoots	Total percentage of phytosterol	Amount in grain planted (0·031 %) in g.	Remarks
Normal Wheat Plants	shoots	500	1950	2250	0·8802	0·9932	0·045	0·04	0·155	Increased during growth
	roots		300		0·1130		0·037			
Etiolated Wheat Plants	shoots	500	135·2	203·5	0·0674	0·1529	0·0487	0·075	0·155	About the same as in seed
	roots		68·3		0·0855		0·1252			
Etiolated Wheat Plants		383		168		0·1175		0·070	0·1187	About the same as in seed

SUMMARY AND DISCUSSION OF RESULTS.

(1) In order to obtain more information as to the relative amounts of phytosterol occurring in the different organs of the plant, those of the wheat plant were carefully examined. Schultze and Barbieri [1882] give some figures for the phytosterol content of the seeds and seedlings of lupin plants, but their methods are open to criticism, and they are not certain of their results. The chief phytosterol present in the grain was found to be the same

as that in the embryo—sitosterol—and as the solid part of the unsaponifiable ether extract of the grain consisted entirely of this substance, it could be separated and weighed directly. A phytosterol was present in bran, but it differed from sitosterol in its melting point, and in the melting points of its esters.

(2) In the case of the vegetative organs, other substances were present in the unsaponifiable ether extract—an alcohol $C_{20}H_{42}O$, which did not give the phytosterol colour reactions and was not precipitated by digitonin, and an oil, and it proved difficult to separate the phytosterol in order to weigh it directly. The researches of Windaus and his co-workers have shown that all the phytosterols hitherto isolated from the higher plants, which have been fully characterised, combine with digitonin to form a compound insoluble in ether, acetone, and ethyl acetate. A method of estimation based on this fact was devised, and it was found to be very accurate when applied to pure sitosterol in preliminary experiments.

(3) An estimation of the amount of phytosterol present in the normal wheat plant, grown from a known amount of grain, shows that the quantity in the adult plant is greater than in the grain, whereas in the etiolated plant the amount is very much the same as that in the grain, allowing for experimental errors. The percentage of phytosterol present in the normal plant is slightly greater than the percentage present in the grain, and in the etiolated plant it is higher still, no doubt correlated with the loss in dry weight, due to starvation.

(4) In the embryo the percentage of phytosterol present is much higher than in the plant. This points to an essential function in germination and growth. As the amount present in the grain is not appreciably altered in the starved plant, there is distinct evidence that this function is *not* a nutritive one. This seems an important point and is rather in agreement with the nature of phytosterol and the fact that it is not easily altered chemically. Its occurrence in the seed, in the seed coat, in the embryo, the root and the shoot, in the case examined, suggest a function common to all of them. While no definite conclusions can be drawn until a larger number of investigations have been made, the theory that the sterols may form an essential part of cell membranes suggests itself, but the presence of phytosterol in latex, unless it occurs there as the result of cell-destruction, and in the form of glucosides, is hardly accounted for by this theory. Many experiments will still have to be carried out before we can form any very definite conclusion as to the function of phytosterol in the plant.

The above experiments were carried out with the help of a grant from the Government Grant Committee of the Royal Society to whom I take this opportunity of expressing thanks.

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XIV. CONTRIBUTIONS TO OUR KNOWLEDGE OF THE PLANT STEROLS. PART II. THE OCCURRENCE OF PHYTOSTEROL IN SOME OF THE LOWER PLANTS.

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(*Received March 4th, 1918.*)

In the first paper of this series [1918], an account of the phytosterols occurring in the lower plants is given. These are represented chiefly by two substances occurring in the fungi: (1) ergosterol, first isolated by Tanret [1889], and (2) fongisterol, which have been obtained by Tanret [1908] and other workers. Gerard [1892, 1895] puts forward the theory that the sterols may be divided into two groups: (1) the Phytosterols occurring in the higher plants, and (2) the Ergosterols found only in the lower plants. To obtain more knowledge on this matter, several species, chosen from different groups belonging to the lower plants, were examined for the occurrence of a phytosterol.

The following were dealt with:

(1) Musci, *Sphagnum.*
(2) Algae, *Laminaria.*
(3) Fungi, (i) *Polyporus nigricans.*
        (ii) *Polyporus betulinus.*
        (iii) *Agaricus rubescens.*
        (iv) *Lactarius subdulcis.*

Method of Experiment.

The plant material is dried, and then ground up very finely in order to render all the tissues permeable.

In the case of the juicy fungi, the material is ground up in a mortar with coarse sand, adding plaster of Paris until the mixture hardens. When it is dry, the mixture is coarsely powdered.

The dried material in either case is then subjected to prolonged extraction with ether in a Soxhlet apparatus. The ether extract so obtained is saponified with a large excess of alcoholic sodium ethylate, according to the method of Kossel and Obermüller. The saponified liquid is always allowed to stand overnight to insure complete saponification. The precipitate of soap is separated by filtration, and washed with ether to remove all the ether-soluble substances. The filtrate is shaken up with water several times to free it from the excess of alkali. Finally the washed ether extract is evaporated to dryness, the residue dried, and examined. Further details will be given for each experiment.

1. *Musci. Sphagnum.* Plants were gathered in the autumn and dried. The dry weight was 622 g.

After saponifying and drying, the ethereal extract showed no sign of crystalline matter, consisting mainly of a brown oil. This oil, however, gave the cholesterol colour reactions.

2. *Algae. Laminaria.* The seaweed was dried in air and "stalks" and fronds were extracted separately. The dry weight of the fronds was 7·132 kilos., and of the "stalks" 1·516 kilos. They were extracted with ether, and the ether extract saponified as usual. Incidentally it was observed that while the ethereal extract from the "stalks" was brown, that from the fronds was green and fluorescent. This points to further modifications in the direction of "stem" and leaf function than the mere external appearance. The pseudo-phloem found in the stalk gives histological support to such a suggestion.

The Fronds. The unsaponifiable extract from the fronds weighed 18 g. It consists mostly of a dark brown oil with a curious penetrating odour. A very small amount of crystalline matter was separated by recrystallisation from alcohol in sheaves of needle crystals, melting at 105°–110°. These gradually turned brown in colour as they were heated from 50°. On further purification with alcohol they melted at 125°.

On standing for some time these crystals seemed to change, lost their form and broke down to an amorphous powder, but the amount was so small that it was impossible to investigate this phenomenon further.

The Stalks. The unsaponifiable extract from the stalks weighed 3·6 g. It consisted almost entirely of a brown oil, only a trace of crystalline matter being separated. This resembled the needle crystals from the fronds.

The bulk of the unsaponifiable matter in both cases consisted of a brown oil which gave the cholesterol colour reactions. This was kept, and from time to time attempts were made to obtain crystalline matter from it from solution in various solvents. It did not apparently change on standing, however, or deposit crystalline matter, and it was thought possible that this oil itself might be a form of sterol. The oil was therefore distilled in steam, when it came over as a pale yellow liquid which darkened very quickly on exposure. This oil gave the cholesterol colour reactions distinctly.

This evidently opens out fresh fields of investigation. Windaus was able to separate the various isomeric forms of dihydrocholesterol by means of digitonin, as they do not all form a compound with this substance, and it is thought that this fact gives a suggestion in regard to the separation of these plant sterols.

The oils from this and other material are being kept. Their examination and identification obviously entail somewhat lengthy investigation, but it may lead to important discoveries in regard to the occurrence of sterols.

3. *Fungi.* (i) *Polyporus nigricans.* The *Polyporus* was gathered in the autumn. The total dry weight of fungi was 1·1 kilos., which gave an unsaponifiable ether extract of about 40 g.

The fungi were extracted in two portions. The first portion of 1050 g. gave an unsaponifiable ether extract weighing 9·6 g. This residue consisted of a dark brown oil, mixed with crystalline matter. The whole was dissolved in 95 % alcohol. (1) The first crop of pure white crystals melted at 248°–249°; on further recrystallisation the M.P. was 250° and this remained constant. The substance was obtained from alcohol in beautiful sheaves of needle crystals. They did not give the cholesterol colour reactions, and were not investigated further at the time. The mother liquors yield a little more of this substance, not quite pure.

(2) The bulk of the more soluble substance, however, melted from 158°–167° and finally a substance giving a constant melting point of 163° was obtained. This was converted into the acetate which melted at 158°. This proved to be a mixture, however, and the author succeeded in separating the constituents by their different solubility in acetone. The less soluble substance melted at 185° after several recrystallisations, and was identified as ergosteryl acetate; this yielded ergosterol, M.P. 165°, on saponification. The acetate of fongisterol, M.P. 158·5°, remained in solution, and was obtained from the mother liquors. Fongisterol itself melts at 144°.

These substances were further identified by a colour test. Ergosterol and strong sulphuric acid give no colour at the end of a minute, whereas fongisterol colours red, then red-violet at the end of a few seconds, so that the crystalline sterols in this *Polyporus* were identified as a mixture of ergosterol and fongisterol—described by Tanret in other fungi.

A quantity of heavy brown oil was left and for some time no more crystalline matter could be extracted. On standing, however, for a long period, it was found that the residues yielded more crystalline matter, similar to the mixture described above, when dissolved in glacial acetic acid. Alcohol, acetone and ethyl acetate were not found to produce the same result.

The residue consists of a heavy, almost clear brown oil, which gives the cholesterol colour reactions, and is being kept for future investigation.

In the case of the second portion of the fungi, the unsaponifiable residue was a sticky, gummy, yellow substance. This dissolved in ether, alcohol, acetone, ethyl acetate, and acetic acid, but came out of solution in the same form and no crystalline matter was obtained.

For some time, all efforts to crystallise this substance failed, but, after it had stood for a considerable period, a further attempt was successful, and the solution in acetone deposited crystalline matter identical with that already described, a less soluble substance melting at 250° and the more soluble mixture of ergosterol and fongisterol.

This seems to give an indication that these bodies may change on standing, from one form to another, or that some process of oxidation occurs on standing.

(ii) *Polyporus betulinus*. The fungi were gathered in the autumn, dried, ground up, and extracted with ether. The weight of fungi was 950 g. which gave an unsaponifiable ether extract weighing 5·48 g.

The extract contains a very little crystalline matter, which melts at 157° and gives a colour test with strong sulphuric acid resembling that of fongisterol, blood-red, then red-violet at the end of a few seconds. It is probably a mixture like that occurring in the other fungi, but the amount was too small for separation of the constituents.

The main bulk of the unsaponifiable ether extract consists of a heavy brown oil, which gives the cholesterol colour reactions. It did not yield any further crystalline matter after standing a considerable time.

(iii) *Agaricus rubescens* (5·5 kilos.) and (iv) *Lactarius subdulcis* (3·5 kilos.) were also examined. The material, consisting of young plants, was ground up with sand and plaster of Paris and allowed to dry. The ethereal extract was saponified and the unsaponifiable ether extract obtained in the usual way. This showed no sign of crystalline matter in the case of either fungus. The residues consisted of brown oils which, however, gave the cholesterol colour reactions.

SUMMARY OF RESULTS.

(1) From the fungus, *Polyporus nigricans*, and probably also from *Polyporus betulinus*, a mixture of two sterols previously known to occur in the fungi—ergosterol and fongisterol—was isolated.

(2) There is a certain amount of evidence that in the lower plants at least, the sterols may occur in a non-crystalline form, as in the alga—*Laminaria*—an oil was obtained by distillation in steam, which gave the cholesterol colour reactions, and the unsaponifiable ether extracts from *Sphagnum* (Musci), *Agaricus rubescens* and *Lactarius subdulcis* (Fungi) consist of brown oils, giving these tests. Also on standing the substances seem to change from one form to another. In *Polyporus nigricans* the unsaponifiable ether extract was at first gummy and sticky and no crystalline matter could be isolated. After standing for some time, however, crystals were obtained from solution in acetone.

In conclusion the author would like to express her grateful thanks to Professor Waller, and particularly to Mr J. A. Gardner, for the facilities afforded.

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XV. A STUDY OF SOME BIOCHEMICAL COLOUR TESTS. No. 1. THE THIOPHENE TEST FOR LACTIC ACID. A COLOUR TEST FOR ALDEHYDES.

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(*Received March 12th, 1918.*)

A characteristic of biochemistry is its great heritage of colour tests which have been devised to meet the demand for means of identifying substances of complex or unknown constitution where the more exact methods of pure organic chemistry were of no avail.

Some of these biochemical tests are of a degree of delicacy and specificity almost comparable to biological reactions; but, owing to the difficulties of micro-analysis, the mechanisms of many of these tests are as yet imperfectly understood.

This is not a practical disadvantage as long as the specific nature of the test is assured, but occasionally doubtful cases arise when the knowledge of the rationale of a reaction is essential, and it was from experience of such cases that I was led to investigate some of the well-known colour tests, laying stress on the nature and limiting conditions of the test apart from the chemistry of the substances involved in it.

Hydrocnic Colour Reactions.

Many colour tests are only obtained in the presence of an excess of strong acid, generally sulphuric. In some cases, addition of water after the colour has appeared merely dilutes the tint, but there are certain colour reactions the products of which lose their colour at once if a few drops of water be added.

I have ventured to suggest the term *Hydrocnic*, derived from "Hudōr" (water) and "Okneō" (I shrink), to describe these tests in which the colour is unstable towards water.

These reactions have several points in common, even when they are due to very dissimilar compounds; such as in the Hopkins lactic acid test, Salkowski's cholesterol test, and many of the aldehyde reactions for tryptophan.

A study of *hydrocnic* phenomena calls attention to various important factors capable of modifying or inhibiting colour reactions, as, for example, the local heating due to the addition of the concentrated acid, and the significance of certain impurities in the acids employed.

The Thiophene Test for Lactic Acid.

This test has been selected as it is a typical *hydrocnic* reaction and, though it involves no complex chemical substances, its mechanism has not yet been fully explained.

The test was introduced by Hopkins and was first described in Cole's *Physiological Chemistry* in 1904.

The test is very sensitive to the presence of water in two ways. If the sulphuric acid used be at all dilute or a little water be added with the lactic acid, the solution on warming becomes yellow and does not give the cherry-red colour on the addition of the thiophene. Again, if a few drops of water be added after the red colour has developed it is at once discharged.

In his description of the test, Hopkins [Hopkins and Fletcher, 1907] observes that it is also given by acetaldehyde and by glyoxylic acid.

It is well known that lactic acid when heated with sulphuric acid is decomposed into acetaldehyde and formic acid, or, if an oxidising agent be present, into pyruvic acid and water.

$$(1)\quad CH_3 . CH(OH) . COOH \longrightarrow CH_3CHO + HCOOH.$$

$$(2)\quad CH_3 . CH(OH) . COOH + O \longrightarrow CH_3 . CO . COOH + H_2O.$$

G. Denigès [1909] has employed alcoholic guaiacol to show this formation of aldehyde, and Ryffel [1909] has used it for the estimation of lactic acid in urine.

The results of the action of sulphuric acid in varying proportions on lactic acid were investigated.

Expt I. 20 cc. lactic acid and 20 cc. pure concentrated sulphuric acid, with a crystal of copper sulphate, were mixed. The liquid gradually darkened and gas was evolved. When the reaction had subsided the mixture was distilled under reduced pressure, from a water-bath at 80°.

The distillate was collected in 20 cc. water. It consisted of formaldehyde, acetaldehyde chiefly, formic acid, acetic acid. The gas evolved was CO_2 with some CO.

Expt II. 10 cc. lactic acid and 20 cc. sulphuric acid as before. This time there was a considerable amount of acetaldehyde in the distillate. Not much gas was evolved.

Expt III. 20 cc. moist lactic acid (containing about 10 % water) and 10 cc. sulphuric acid. The distillate contained only a trace of aldehyde but gave a fairly distinct red coloration with ferric chloride.

There was a great evolution of gas (chiefly CO) during the reaction.

The thiophene lactic acid test was then performed on a drop of each of the distillates. I and II gave the cherry-red, III gave a faint orange.

Each of the substances identified in the distillate was then tested separately, when it was found that both formaldehyde and acetaldehyde gave a colour with thiophene in the presence of excess of sulphuric acid.

With regard to the poor production of aldehyde in the last experiment, it was found that acetaldehyde is much less stable in moderately dilute sulphuric acid than in strong acid, consequently it is necessary in performing the lactic acid test to see that there is no appreciable amount of water present at the outset, otherwise the acetaldehyde will be destroyed as it is produced and the thiophene will give no coloration.

It is this decomposition of aldehyde that probably accounts for the 10 % error in Ryffel's method for estimating lactic acid in urine.

A Colour Test for Aldehydes.

Working along the lines of the previous test, the following reaction for aldehydes was devised.

A couple of drops of a 0·2 % alcoholic solution of thiophene are added to 5 cc. concentrated sulphuric acid (which must be free from nitrous and nitric contaminations) and mixed. Then, if a drop of a weak solution of an aldehyde be added, a red colour develops and spreads through the acid. A few drops of water discharge the colour but it returns on the addition of more sulphuric acid.

The test is very delicate with most aldehydes. 1 part CH_2O in 10,000 parts H_2O gives a deep plum-coloured ring: 1 part in 100,000 gives a distinct band.

The colour varies slightly with the different aldehydes. Formaldehyde gives a purple red, acetaldehyde a cherry-red, acrolein a rose carmine. The test is given also with the substituted aldehydes, such as chloral, *p*-hydroxybenzaldehyde, etc. All these colours are typically hydroenic.

By the aid of this test, the presence of aldehydes in moist ether can be demonstrated. If some aqueous ether be left in the sunlight for a few days it gives an aldehyde reaction with the sulphuric acid and thiophene. This fact is of considerable importance since ether is frequently used in the extraction of lactic acid. The ether employed should be fresh and dry and the subsequent evaporation should not be done on a water-bath but at ordinary temperature. It is well known that moist ether, when warmed, readily oxidises with production of acetaldehyde and other substances.

The Interaction of Thiophene and Aldehydes.

Amongst the early work on thiophene two condensations with chloral and bromal, in the presence of glacial acetic acid and sulphuric acid, are recorded by V. Meyer [1883] and A. Peter [1884]. The formula ascribed is

$$CCl_3 . CH : (C_4H_3S)_2 .$$

for the chloral derivative.

Töhl and Nahke subsequently [1896] described a condensation between thiophene and benzaldehyde with the formation of dithienylphenylmethane;

$$C_6H_5 . CH : (C_4H_3S)_2 .$$

On mixing thiophene and acetaldehyde in light petroleum in the molecular proportions corresponding to a body, $CH_3 . CH : (C_4H_3S)_2$, and adding phosphorus pentoxide, the pentoxide turned deep purple and a reaction began. The products were extracted with alcohol, ether, and light petroleum, respectively. The extracts were neutralised, washed, dried and fractionated, or fractionated straight away.

From two of the extracts a liquid fraction was obtained which gave a deep crimson with sulphuric acid.

The fraction was an orange liquid which boiled with decomposition at 121°, was insoluble in water, and had a peculiar smell.

The determination of the molecular weight and the composition of this substance was deferred until an adequate supply of thiophene could be obtained.

SUMMARY.

1. It is proposed to introduce the term *hydrocnic* for biochemical colour reactions, the products of which are decolorised by the addition of small quantities of water.

2. The thiophene reaction for lactic acid is due to the production of formaldehyde and acetaldehyde from the lactic acid, which interact with the thiophene in the presence of excess of sulphuric acid to give the cherry-red colour.

3. A new colour test for aldehydes is described depending on the fact that they interact with thiophene in the presence of sulphuric acid to give coloured substances.

4. A preliminary reference is made to the reaction between aldehydes and thiophene.

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XVI. THE ROLE OF THE LIVER IN HIPPURIC ACID SYNTHESIS.

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The attempt will be made in the following pages to show that by the use of hydrazine, a specific agent affecting liver tissue and leaving that of the kidney intact [Wells, 1908; Underhill and Kleiner, 1908], it is possible to demonstrate a considerable participation of the liver in hippuric acid conjugation. The studies of Bunge and Schmiedeberg [1876], followed by the corroborating studies of Kochs [1879] and of Bashford and Cramer [1902], have been interpreted as decisive evidence of a difference in power of effecting hippuric acid pairing on the part of carnivores, including man, and herbivores, in that the seat of the process in herbivores is not limited to the kidney as in the case of the carnivores. This conclusion remained practically unchallenged until Kingsbury and Bell [1915, p. 301] found "a relatively large amount of hippuric acid in the liver of a nephrectomised dog after injection of glycocoll and sodium benzoate" suggesting "that the liver like that of the rabbit may possess this synthetic power."

In the present set of experiments we have departed from the procedure followed by other investigators in two respects, namely, that we have utilised the urine rather than the blood, and secondly, we have employed a reagent which may be used subcutaneously to cause liver involvement while leaving the kidney intact without introducing the additional factor of operation, ligaturing, etc.

METHODS.

Two male dogs, weighing respectively 9·05 and 9·50 kg., were isolated in metabolism cages. The urine was collected in 24 hour quantities, thymol in alcohol being used as a preservative; faeces were excluded by use of glass-wool and filter plates. The food consisted of dog-biscuits supplemented by fresh beef, chicken trimmings and boiled meat from the soup kitchen. The intake of water and of chloride was noted; likewise we noted carefully the general condition of the dogs especially after the administration of reagents. The dogs were kept in the cages several days before the collection of urine was commenced in order that they might become accustomed to their surroundings and also that their controlled food might replace that previously given them.

In the administration of sodium benzoate (2 g.) and of hydrazine sulphate (500 mg.) by mouth, we wrapped the substances in fresh steak formed into a bolus which the dogs swallowed freely, thus insuring that the animals received the complete amount of the drug. Subcutaneous injections of 500 mg. hydrazine sulphate dissolved in 20 cc. of sterile water were given into the flanks under aseptic conditions.

Hippuric acid was determined in 50 cc. aliquots of urine after the method of Folin and Flanders [1912], the reduction in amount of urine from 100 cc. to 50 cc. being compensated for by reducing the amounts of the other reagents, nitric acid, ammonium sulphate, sodium chloride, chloroform, etc., correspondingly.

Chlorides were determined by the Volhard method, for renal efficiency. Amino-nitrogen was determined gasometrically upon the hydrolysed urine. Free benzoic acid was determined upon the residue from 50 cc. of evaporated urine; the dry residue was transferred to an allundum thimble and extracted in a Soxhlet apparatus for four hours with chloroform as solvent. Titration was then performed with decinormal sodium ethylate from sodium, as in the case of the hydrolysed hippuric acid determinations. On the thirty-fourth day the dogs were killed by means of coal gas and autopsied.

RESULTS.

The following table gives the results for the two dogs extending over a period of 34 days; the quantities are calculated for 24-hour periods:

Day	Dog	Hippuric N.	Amino N.	NaCl	24 hour quantity urine	
1	1	0·014 mg.	0·035 g.	1·45 g.	150 cc.	
	2	0·023	0·749	0·96	130	
2	1	0·026	4·190	3·07	360	
	2	0·049	2·120	0·28	300	
3	1	None	None	None	None	
	2	0·029	,,	0·58	100	
4	1	0·014	0·190	5·90	100	
	2	0·040	0·888	0·25	550	
5	1	0·010	0·190	1·40	150	
	2	0·026	0·526	0·19	275	
6	1	None	None	None	None	
	2	0·119	2·15	0·39	500	
7	1	0·083	3·51	2·80	500	
	2	0·037	0·800	0·25	500	
8	1	None	None	None	None	2 g. sodium benzoate
	2	,,	,,	0·12	400	

Day	Dog	Hippuric N.	Amino N.	NaCl	24 hour quantity urine	
9	1	0·174	3·05	3·20	425	
	2	0·091	None	0·51	225	
10	1	0·030	1·05	1·70	250	
	2	0·076	0·945	0·28	450	
11	1	0·054	1·91	2·10	230	
	2	0·040	0·314	0·27	260	
12	1	0·058	2·22	3·50	400	
	2	0·074	1·860	None	380	500 mg. hydrazine sulphate per os.
13	1	None	None	,,	None	
	2	,,	,,	0·17	375	
14	1	,,	1·70	1·40	250	
	2	0·089	1·97	0·26	400	
15	1	0·010	0·124	1·20	140	
	2	0·132	0·895	0·26	500	
16	1	0·100	1·43	3·50	450	
	2	0·29	1·87	0·14	260	
17	1	None	None	None	None	
	2	0·017	0·776	0·16	250	
18	1	0·071	None	3·20	450	
	2	0·026	,,	0·15	280	
19	1	None	0·22	3·60	500	
	2	0·020	0·717	0·22	400	500 mg. hydrazine sulphate per os.
20	1	0·010	1·00	None	150	
	2	0·013	1·134	,,	160	
21	1	None	None	0·50	200	
	2	,,	,,	0·12	180	
22	1	0·018	,,	0·51	150	
	2	0·028	1·05	0·41	150	
23	1	0·024	1·37	None	180	
	2	0·024	None	,,	350	
24	1	None	,,	0·82	130	
	2	,,	,,	0·18	150	
25	1	0·036	,,	0·37	450	
	2	None	,,	0·21	250	500 mg. hydrazine sulphate sub-cutan.
26	1	0·016	0·504	0·92	250	
	2	0·018	1·091	0·37	150	2 g. sodium benzoate
27	1	0·099	1·74	0·45	250	
	2	0·051	1·728	None	250	
28	1	0·020	1·740	0·26	200	
	2	None	None	0·13	225	
29	1	0·028	2·33	None	375	
	2	0·015	0·981	,,	130	
30	1	0·014	0·733	,,	100	
	2	0·042	2·52	,,	325	
31	1	0·010	None	0·83	100	
	2	0·026	2·106	None	300	
32	1	None	None	1·73	350	
	2	,,	,,	0·072	225	500 mg. hydrazine sulphate sub-cutan.
33	1	0·013	0·075	0·75	160	
	2	None	0·114	0·10	200	
34	1	0·017	None	0·43	300	
	2	0·014	0·353	0·07	250	2 g. sodium benzoate

Professor O. T. Schultz, who kindly performed the autopsies and examined the slides from formalin and Zenker fixations of portions of the livers and kidneys of the two dogs, reports that both dogs showed marked fatty degeneration of the liver, while the kidneys were intact.

DISCUSSION.

We may summarise the results by averages of the amounts of the various substances excreted during the different periods of the experiments.

Average of excretions previous to administration of sodium benzoate or hydrazine:

Dog	Hippuric N.	Amino N.	Chlorides	Quantities
1	0·031	1·98	2·92	252
2	0·043	1·17	0·45	332

Average after giving 2 g. of sodium benzoate:

1	0·085	2·00	2·33	301
2	0·069	0·629	0·29	301

Average after giving hydrazine sulphate by mouth:

1	0·059	1·36	2·48	323
2	0·104	1·47	0·019	349

Average after a second feeding of hydrazine sulphate:

1	0·017	0·863	1·35	218
2	0·021	0·969	0·23	231

Average after injection of hydrazine subcutaneously:

1	0·026	0·50	0·64	350
2	0·018	1·091	0·29	200

Average after feeding sodium benzoate following hydrazine injection:

1	0·040	6·54	0·51	203
2	0·033	1·83	0·13	246

Average after a second injection of hydrazine:

1	0·013	0·075	1·24	255
2	None	0·57	0·086	213

Average after a final feeding of benzoate following hydrazine injection:

1	0·017	None	0·43	300
2	0·014	0·353	0·07	250

From these results, we see that following the feeding of sodium benzoate initially after establishing the normal excretion, the hippuric acid nitrogen is increased. This is true after we subtract the free benzoic acid figure from the total amount determined after hydrolysis, namely, 0·026, leaving 0·065 mg in the case of Dog 1. After the feeding of hydrazine by mouth, upon the first occasion, there is no apparent disturbance of hippuric acid excretion, such as we find in the case of the second feeding. There is an apparent reduction of the amount of hippuric acid after the injection of hydrazine subcutaneously, but inasmuch as we find a rise in excretion following the administration of sodium benzoate, we may conclude that no extensive damage has been done to the liver up to this point. After a second injection of hydrazine subcutaneously, there is a marked fall in the case of Dog 1, the figures for the second dog being lost due to defect in the water system passing through the reflux condensers during hydrolysis. Finally, after the feeding of sodium benzoate after the second injection of hydrazine, a marked fall in hippuric acid excretion is observed. Obviously, we are not able to say how great a difference in liver involvement has occurred between the twenty-fifth day and the final day of the experiment, but from the figures, it is evident that the damage has been progressive.

During these periods, the kidneys have remained intact, as we may observe from the chloride excretion; in studying these figures, it must be borne in mind that during the last periods of the experiments, the dogs took less and less food, so that the chlorides appeared in progressively smaller amounts. However, a comparison of the figure for the chlorides excreted by Dog 1 during the first period and during the next from the last, namely, a comparison of 2·92 g. with 1·24 g., makes it evident that after it has become established that the liver is damaged, the kidneys are excreting quantities of chlorides not far from what we should expect, taking into consideration the lessened intake in the food. The chemical figures are fully borne out by the study of the histological sections.

We were disappointed in finding that the amino-nitrogen could not be correlated with the hippuric acid figures. At best, the glycocoll fraction is not large and might easily be overbalanced by changes in the amino-nitrogen fraction due to the degenerative changes of the liver. Henriques and Sörensen [1909] have utilised the glycocoll fraction after hydrolysis of hippuric acid, the determination being made by means of the formaldehyde-titration method.

From the figures representing the 24 hour quantities of urine, it is apparent that there is no effect either as diuretic or anuretic exerted by the reagents used in the experiments. Consequently, the results are not vitiated by these factors, which Astolfoni [1905] found operative in his experiments, hippuric acid being excreted in increased amounts following the administration of diuretics and vice versa.

The figures obtained upon free benzoic acid in the urine show that there is no effect upon the relative amounts of hippuric acid estimated as benzoic acid after hydrolysis, by this factor, so that the criticism of Lewiniski [1908] cannot be applied to this work.

The conclusion that the liver plays an important role in hippuric acid synthesis is well in keeping with what we know of the functions of the liver, as contrasted with those of the kidney. There are a number of conjugations performed in the liver. Thus, various syntheses are known involving the acids, such as phosphoric acid, sulphuric acid, glycuronic acid, etc. Conjugations with sulphuric acid lead to the formation of the so called ethereal sulphates; pairing of glycuronic acid with substances of various sorts leads to the formation of glucoside compounds. Conjugation of glycocoll occurs with any compound which after oxidation leads to the formation of benzoic acid (salicylic acid, *p*-hydroxybenzoic acid, etc.), the end-product being the so called "ur-acids" of acid-amino nature. In birds, the formation of dibenzoylornithine, where ornithine takes the place of glycocoll in hippuric acid syntheses, is known to take place principally in the liver. In the dog, a subsequent pairing of hippuric acid and urea takes place in the liver. This does not necessarily show that the primary conjugation to form hippuric acid occurs in the same organ as the secondary pairing, yet there is reason to believe that this is the case. The alleged hippuric acid synthesis is the exception to the rule concerning one of the characteristic functions of the liver, if we adopt the conclusions of Bunge and Schmiedeberg and their school as final.

CONCLUSIONS.

Data are presented to show that the liver participates in hippuric acid conjugation to a marked extent. The data have been derived by poisoning the liver with hydrazine, leaving the kidneys intact. The conclusions reached in this investigation are borne out by the work of Kingsbury and Bell and are aligned with our knowledge of the characteristic synthetic functions of the liver as contrasted with those of the kidney.

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XVII. ON THE CONCENTRATION OF ANTITOXIC SERA BY THE SALTING OUT OF THE HEAT-DENATURATED SERUM PROTEINS WITH SODIUM CHLORIDE.

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(*Received April 17th, 1918.*)

A recent investigation of the factors influencing the concentration of antitoxic sera by methods involving the use of ammonium sulphate has shown that the degree of concentration attained is regulated by the extent of the denaturation of the serum proteins induced during the heating of the serum. There is, however, a limit to the degree of concentration. In cases where the heat-denaturation is greater than about 50 %, the attempted separation of the antitoxin from the heated sera by means of ammonium sulphate leads to the production of end products showing a considerable loss of antitoxin. The missing antitoxin is carried down with the First Fraction precipitates (i.e. at 30 % of saturation with ammonium sulphate) and for its recovery it is necessary to extract those precipitates with brine [Homer, 1917, 1].

Even in our routine work where the heat-denaturation of the serum proteins is of the order of 30 %, we have found it advisable, for reasons based on the above observations, to resort to the extraction of the First Fraction precipitates with brine. As the adoption of this procedure necessitates the use of two "salting out" agents it seemed worth while to ascertain whether the process of concentration could be shortened by treating the heated sera directly with sodium chloride, thus dispensing with the use of ammonium sulphate.

In this connexion it was necessary to make a further study, not only of the factors influencing the heat-denaturation of the serum proteins, but also of the extent to which the denaturation can be carried without rendering the protein-antitoxin complex insoluble in brine. The work, which is still in progress, has shown that the extent of the heat-denaturation of the serum proteins depends on:

(1) The reaction of the serum or plasma previous to its being heated [Homer, 1917, 2].

(2) The temperature at which the heating is conducted.

In all cases the maximum values are more quickly reached in acid than in alkaline sera and, irrespective of the $[H^{\cdot}]$ of the sera, the time taken is far less at high than at low temperatures.

It was also found that the maximum values for the heat-denaturation at a specific reaction and at a specific temperature could be increased by the addition of substances such as phenol and its homologues, ether, chloroform, or electrolytes to the serum previous to its being heated.

The knowledge thus gained has been applied to the present investigation. Pending the completion of a series of experiments dealing with the effect of heating sera at temperatures ranging from 58 to 72° on the extent of the heat-denaturation of the serum proteins, and the bearing of the latter on the solubility of the thus denaturated proteins and on the inactivation of antitoxin, my experiments have been conducted at temperatures between 57° and 58°, for, it has been shown that during the prolonged heating of sera at this temperature there is no actual destruction of antitoxin even in those cases in which an extensive heat-denaturation of the serum proteins has been induced.

In order to ensure the desired heat-denaturation, the plasma, previous to being heated at 57–58°, was subjected to one of the following methods of treatment:

(I) The reaction was suitably adjusted by the addition of acid or alkali:

(II) To the plasma was added 2 % of sodium chloride followed by fixed amounts of phenol or its homologues; or

(III) To the plasma were added various amounts of electrolytes.

The treated plasma was then heated at 57–58° for the necessary time. At the end of the heating period (*vide infra*) there were added to the hot serum twice its volume of a saturated solution of sodium chloride and a sufficient amount of solid salt to ensure complete saturation of the brine serum mixture[1]. The liquids were allowed to stand at room temperature for several days.

It was found that after the mixtures had stood for two to three days there was then, practically speaking, a complete extraction of the antitoxin from those sera which, during the heating, had remained clear or had become opalescent, or of which the consistency had been changed into that of a thin suspension unfilterable through paper. In those cases in which the consistency of the serum had been changed to that of a thick suspension, due to the actual precipitation of particles of heat-denaturated protein, a much longer time was required for the extraction of the antitoxin by the brine. Thus, with the sera showing a heat-denaturation of 40 to 45 %, about 6 to 8 days' extraction was sufficient for the extraction of the antitoxin. On the other hand, even after several weeks' extraction, the recovery of antitoxin was far from complete from those sera in which a more extensive heat-denaturation and correspondingly increased precipitation of heat-denaturated protein had been induced.

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[1] Tested by means of a hydrometer.

After the brined liquids had stood at room temperature for the necessary length of time they were filtered. Except where phenol and cresylic acid had been added, there was slow filtration of the brined extract of those sera of which the reaction, previous to the heating, lay between P_H 5·5 and 7·2. The slow filtration was due to the presence of heat-denaturated protein in a semi-gelatinous condition. The difficulty in filtration was obviated by gradually warming the liquids to a temperature of 45°, thereby inducing the coagulation of the particles of protein into aggregates of sufficient dimensions to enable their separation to be effected by filtration through paper.

The precipitates were washed with brine and the washings, after filtration, were added to the main bulk of the filtrates to which was then added 0·25 % of its volume of glacial acetic acid. The ensuing precipitate was filtered, drained, pressed and after the addition of 3 % of washing soda was dialysed until the salt content was the same as that of the water in the dialysing tank. To the residue from dialysis was added 1 % of sodium chloride and 0·35 % of cresylic acid.

The end products were clear and readily filterable, for, in contrast to those obtained by methods involving the use of ammonium sulphate they were not liable to contain particles of protein in colloidal suspension.

On the other hand they showed a lower percentage removal of the serum proteins and a consequent lower degree of concentration than is now obtained in the Banzhaf [1913] and the Homer [1916] methods.

The less complete removal of the serum proteins can be attributed mainly to the fact that the addition of 0·25 % of glacial acetic acid to the brined sera leads to the precipitation of a considerable amount of albumin with the pseudoglobulin. As the antitoxin is attached to the latter, the presence of the former must reduce the degree of concentration to a value considerably less than would have been obtained had the precipitation of albumin at this stage been avoided.

The observations that were made during the progress of the work have presented further points of interest with regard to the properties of heat-denaturated serum proteins. They are dealt with in detail below and may be of assistance to those engaged in problems connected with the concentration of sera.

I. THE SALTING OUT OF SERA IN WHICH THE HEAT-DENATURATION HAS BEEN REGULATED BY AN ADJUSTMENT OF THE REACTION.

The hydrogen ion concentration $[H^{\cdot}]$ was adjusted to values ranging from P_H 8·7 to 4·2 by the addition of the necessary amounts of ammonia, acetic or sulphuric acids respectively, the determinations being made in a Walpole's colorimeter with the indicators suitable for the purpose [Homer, 1917, 4].

The alkaline sera were heated to a temperature of 57–58° for a period of not less than four hours; the acid sera were kept at this temperature for two hours only.

At the conclusion of the heating the amounts of acid or of alkali initially added to the sera were respectively neutralised. The liquids were then treated with solid sodium chloride and with brine in the manner described above.

It was found that the success of the operation was influenced by the nature of the changes induced during the heating of the serum. As the extent of the change was primarily regulated by the $[H^{\cdot}]$, comparisons were made of the results from the concentration of those sera of which previous to their being heated the $[H^{\cdot}]$ had been adjusted to values between P_H 8·7 and 5·0, 5·0 and 4·7 and to values more acid than P_H 4·7.

(*a*) *The concentration of sera of which the* $[H^{\cdot}]$ *had been adjusted to values between* P_H 8·7 *and* 5·0.

In Table I have been embodied the results obtained from the concentration of such sera.

It will be seen that, where the $[H^{\cdot}]$ had been adjusted by the addition of acetic acid or of ammonia, there was a sufficient recovery of antitoxin in the final products to warrant the application of the method to the routine concentration of sera. As was to be expected a further recovery of antitoxin could be made by a second extraction of the precipitates with brine.

However, in those cases in which sulphuric acid had been employed to adjust the $[H^{\cdot}]$ to values between P_H 5·2 and 5·0, the recovery of antitoxin in the end product was considerably less than where the reaction had been adjusted approximately to the same values by the addition of acetic acid. It was found that, although, in the two sera, the extent of the heat-denaturation was approximately the same, the percentage removal of the serum proteins was considerably more from those sera acidified with sulphuric acid than from those to which acetic acid had been added.

TABLE I.

Showing the results obtained from the concentration of antitoxic sera by the direct treatment of the heated sera with salt. The extent of the heat-denaturation had been regulated by an adjustment of the $[H^{\cdot}]$ *to values between* P_H 8·7 *and* 5·0.

Reaction of the plasma adjusted by the addition of:—	P_H of the plasma previous to its being heated	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage of the total proteins of the original serum removed during the process of concentration	Percentage of the total antitoxic units of the original serum recovered in the residue from the dialysis of the proteins precipitated by the addition of acetic to the filtered brined sera	Increased potency per c.c. shown by the end products calculated, for the purpose of comparison, on the basis that they contain 20 °/o of protein
Acetic acid	5·4	16	26	—†	—
,,	5·2	27	35	> 90	4
,,	5·15	30	40	—†	—
,,	5·1	33	43	—†	—
,,	5·08	36	43	86	4·5
,,	5·05	40	58*	75	4
,,	5·00	43	54	88	5
Sulphuric acid	5·18	33	54	65	5
,,	5·05	43	80	< 50	< 3
Ammonia	8·3	35	46	84	5
,,	8·5	40	55	88	5
,,	8·7	42	54	—*	—

* Brined plasma which had stood at room temperature for two days only.

† Not tested owing to the scarcity of experimental animals.

It is thus evident that there are factors other than the $[H^{\cdot}]$ which affect the nature of the changes induced during the heating of sera. It is possible that the above increased precipitation was due to the more marked effect of the divalent "sulphions" than of the monovalent "acetions" on the *electrical* relationship between the heat-denaturated protein and the solution; a relationship which is recognised as a determining factor in the precipitation of a colloid.

(*b*) *The concentration of sera of which the* $[H^{\cdot}]$ *had been adjusted to values between* P_H 5·0 *and* 4·7.

In these cases the consistency of the heated sera was that of a thick suspension due to the precipitation of heat-denaturated protein in a filterable form.

It was ascertained that, during the process of concentration, the recovery of antitoxin in the end products decreased as the acidity of the sera was increased. At the same time there were indications that the recovery in brine was more satisfactory from those sera in which the reaction had been adjusted by means of acetic acid than where sulphuric acid had been used.

Thus, it was found that with sera acidified with acetic acid, and showing a heat-denaturation of about 50 %, the extraction of the heated sera with brine for two days led to the recovery of about 50 % of the antitoxin; after seven days the recovery amounted to 66 %. Where the heat-denaturation had been of the order of 70 %, the treatment of the heated sera with brine for seven days led to the recovery of about 30 % of the antitoxin. In neither case was there a further increase in the percentage recovery of the antitoxin by a prolongation of the time of extraction to a period of five weeks.

On the other hand with sera acidified with sulphuric acid, in which a heat-denaturation of 35 to 40 % had been induced, there was a recovery of only 66 % of the total antitoxin after seven days' extraction with brine. Where the denaturation had been of the order of 65 % the recovery was less than 10 % in the same time. In neither case was the extent of the recovery of the antitoxin appreciably improved by a prolongation of the time of extraction to five weeks.

The more marked insolubility of the heat-denaturated proteins in brine induced during the heating of sera to which sulphuric acid had been added, is shown by the following experiment.

The reaction of antitoxic plasma was adjusted by means of acetic acid and of sulphuric acid respectively so as to ensure a heat-denaturation of about 60 %. The adjusted sera were then heated for two hours at 57–58° by which time they had become semi-solid in consistency. In each case after the neutralisation of the acid there was added an equal volume of a saturated solution of sodium chloride, and the mixture was well shaken and filtered at once. The precipitates were well drained and, after being freed from adhering fluid by gentle pressure between cloths and boards, were triturated in a mortar with 50 cc. of a saturated solution of salt. The thin paste was then diluted with the salt solution to a volume six times that of the original plasma. The liquids were kept at room temperature in stoppered flasks and were well shaken from time to time.

After standing for stated periods of time, samples of the liquids were filtered and the protein and antitoxin contents of the filtrates were measured. The results showed that at the end of one week the brine had redissolved an appreciable amount of the heat-denaturated protein precipitated from those sera to which acetic acid had been added. Animal tests showed that, to this redissolved protein, was attached about 50 % of the total antitoxin taken for concentration. The amount of protein and antitoxin thus recovered from the precipitate was not appreciably increased after a further twenty-one days' extraction.

On the other hand none of the filtered brine extracts of the protein precipitated from the sera acidified with sulphuric acid contained more than a trace of protein even after four weeks' extraction; the antitoxin recovered was considerably less than 10 % of the original.

(*c*) *The concentration of sera of which the* [H˙] *was more acid than* P_H 4·6.

To separate volumes of antitoxic sera were added the requisite amounts of acetic and of sulphuric acids respectively to bring the reaction to the value P_H 4·4. The sera were then heated for two hours at 57–58°, and, after the neutralisation of the acid, the semi-solid liquids were treated with salt and with brine in the manner described above. Samples of the brined sera were taken at stated periods and the antitoxic content of the filtrates were compared.

After ten days traces only of the protein had been redissolved; the amount of antitoxin in solution was considerably less than 10 % of the original. There was no improvement in the recovery of antitoxin effected by a prolongation of the time allowed for extraction to four weeks.

The precipitated heat-denaturated proteins from the sera thus acidified, were triturated with brine in the manner described above. Even after several weeks' extraction in a volume of brine six times that of the original serum, there was redissolved practically none of the protein precipitated from the sera acidified with sulphuric acid, and not more than a trace where acetic acid had been used. In both cases the filtered brine extracts contained considerably less than 10 % of the original antitoxin of the serum.

The observations recorded under (*a*), (*b*) and (*c*) thus indicate that there is a greater production of "salt-insoluble" protein during the heating of sera of which the [H˙] has been adjusted to values more acid than P_H 5·4 by the addition of sulphuric acid than by that of acetic acid. They also demonstrate that, irrespective of the acid used, an increase in the degree of acidity is followed by an increase in the precipitation of heat-denaturated protein and a corresponding decrease in the recovery of antitoxin in a form in which it can be rendered suitable for use clinically.

It is thus evident that, for the successful concentration of sera by the method under discussion, it is advisable to adjust the reaction of the plasma to values about P_H 8·3 or about 5·0. For this purpose a routine method has been suggested [Homer, 1917, 4] in which use is made of the indicators *α-naphtholphthalein* and *dibromo-o-cresolsulphonephthalein*.

Since slight variations in the reaction round about P_H 8·3 are not followed by the marked variations in the heat-denaturation and precipitation of the serum proteins so characteristic of sera more acid than P_H 5·5, preference has been given to the adjustment of the $[H^{\cdot}]$ to the former value.

II. THE "SALTING OUT" OF SERA IN WHICH THE HEAT-DENATURATION HAD BEEN CONDUCTED IN PRESENCE OF CRESYLIC ACID.

As the use of trikresol[1] has been of such practical value in improving the concentration of sera by the Banzhaf [1913] and the Homer [1916] processes, experiments were undertaken to ascertain whether its use in the method under consideration would be followed by similar satisfactory results.

Experimental concentrations were carried out as follows.

To plasma containing 2 % of solid sodium chloride were added fixed amounts of cresylic acid. The reaction of the plasma was then adjusted to values ranging between P_H 7·2 and 8·5 previous to its being heated. The heated plasma, after being treated with solid salt and with brine in the manner described above, was allowed to stand at room temperature for at least six days to ensure the extraction of the antitoxin.

It was found that the antitoxin could be completely recovered by the brining of sera containing 0·35 % of cresylic acid or less which had been heated to 57–58° for periods of from four to six hours. The addition of cresylic acid in a greater amount led to an increased production of "salt-insoluble" protein: during the process of concentration, the antitoxin attached to this insoluble protein was lost. Thus, the filtered brine extracts of those sera containing

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[1] Since the publication of the papers on this subject, we have read in the current literature an abstract of a paper by Banzhaf in which, for the improvement of his technique, he recommends the use of trikresol in an amount somewhat greater than that advocated by us.

We have recently ascertained that the amount of trikresol necessary for the production of clear end products, without inducing undue losses during the process of concentration, depends on the composition of the sample used. Thus with that in use during the years 1916–1918, it was necessary to add 0·30 % to the serum; with a recent consignment of the substance only half this amount was required. It is therefore advisable for those in charge of the concentration of sera to ascertain experimentally, with the trikresol at their disposal, the minimum amount necessary to give the desired results.

In our routine work we have not found it advisable to raise the temperature of the serum mixtures to the high limit now suggested by Banzhaf [1918].

0·5 % of cresylic acid showed a loss of about 15 % of the antitoxin; with 0·75 % of this substance the loss was somewhat greater. The percentage recovery of antitoxin was in no way increased by a prolongation of the time allowed for extraction or by an increase in the volume of brine added to the sera.

Appreciable losses were also experienced in the concentration of sera containing 0·30 to 0·35 % of cresylic acid in which the heating had been conducted at temperatures above 60°.

The losses were, in part, due to the precipitation of antitoxin with the "salt-insoluble" protein and, in part, to an actual inactivation of antitoxin. The extent to which the cresylic acid influences these factors is in the course of investigation and will be dealt with in a future communication.

During the course of the concentration of sera containing cresylic acid it was noticed that, irrespective of the [H˙] of the batches of sera, the brined extracts of the heated sera filtered readily. The ease of filtration was probably due to the stimulating effect of the cresylic acid on the aggregation of the heat-denaturated protein into particles of sufficient dimensions to ensure their separation by filtration through paper.

Table II.

Showing the results obtained from the concentration of sera by the direct treatment of the sera, heat-denaturated in presence of cresylic acid, with salt.

	Percentage of cresylic acid added to the plasma	P_H of the plasma previous to its being heated	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage of the total proteins of the original plasma removed during the process of concentration	Percentage of the total antitoxic units of the original plasma appearing in the residue from the dialysis of the proteins precipitated by acetic acid from the filtered brined sera	Increased potency per c.c. shown by the end products, calculated for purposes of comparison on the basis that these contain 20 % of protein
A	0·00	8·3*	40	46·8	about 85†	5
	0·30	7·4	22·3	51·1	,, 85†	4
	0·30	8·0*	42·0	46·8	,, 85†	4
B	0·00	7·6	18·2	55·4	> 90†	5
	0·25	7·6	36·5	48·0	> 88†	4·6

* Reaction adjusted by the addition of ammonia.

† Not tested out because of the shortage of experimental animals.

The data with regard to concentrations of plasma containing 0·30 % of cresylic acid have been embodied in Table II. They show that, in spite of the increased heat-denaturation induced by the addition of cresylic acid, there was no appreciable increase in the percentage removal of the serum proteins. For this reason the degree of concentration did not show any improvement on that obtained in control experiments with the same sera containing no cresylic acid.

This anomaly was eventually shown to be due to the fact that, while the cresylic acid increases the precipitation and removal of heat-denaturated pseudoglobulin, it also induces an increased precipitation of albumin at the stage at which the pseudoglobulin and antitoxin are precipitated.

III. THE "SALTING OUT" OF SERA IN WHICH THE HEAT-DENATURATION HAD BEEN INDUCED IN THE PRESENCE OF ELECTROLYTES.

It has been demonstrated [Homer, 1916] that there is no appreciable destruction of antitoxin during the prolonged heating of antitoxic sera, to which electrolytes have been added, at 57-58°. As a detailed study has already been made of the effects produced by the addition of definite amounts of sodium chloride to the serum previous to its being heated, attention has been confined in the present investigation to the addition of sulphates to the plasma. For, in view of the more marked tendency of the "sulphion" (*ante*, p. 194) to render the particles of heat-denaturated protein insoluble in brine, it was thought that their addition to the plasma might lead to a greater removal of the serum proteins, and consequent increased degree of concentration, than had been obtained in sections I and II.

Experimental concentrations were accordingly undertaken in which to fixed volumes of the plasma were added definite amounts of the sulphates of ammonium, sodium, potassium, magnesium and zinc respectively. The liquids after being heated at 57–58° for periods of from four to six hours were treated with salt and with brine according to the directions given above; the brined sera were allowed to stand at room temperature for periods of from ten to fourteen days.

The observations made during the progress of the experiments were as follows.

(*a*) *The effect of the addition of sulphates on the changes induced during the heating of the sera.*

In all cases it was evident that the addition of sulphates increased the precipitation of heat-denaturated protein.

With the aid of the Zeiss immersion refractometer determinations were made of the actual precipitation of protein from the heated sera and also of the increased heat-denaturation induced in the presence of the respective sulphates. The results which were compared with those obtained from control experiments with the original plasma have been embodied in Table III.

TABLE III. *Showing the changes induced during the heating of sera containing sulphates.*

Percentage of electrolyte added to the plasma previous to its being heated	P_H of the plasma previous to its being heated	Appearance of the plasma to which the electrolyte has been added (*a*) Before being heated	(*b*) After being heated	Percentage increased precipitation of the serum proteins during the heating of the plasma	Percentage increased precipitability of the serum proteins by 30 per cent. of saturation with ammonium sulphate induced during the heating
$MgSO_4 7H_2O$					
0	7·4	clear	opalescent and slight deposit	2·0	17·7
15	7·4	"	slight precipitate	2·7	17·0
20	7·4	slight precipitate	increased precipitation	3·7	17·7
30	7·4	" "	" "	13·0	24
20 and ammonia	8·0	" "	thin emulsion	28	32·8
K_2SO_4					
0	7·0	clear	opalescent and slight deposit	0	10
5	7·0	very slight precipitate	very slight precipitation	1	11
10	7·0	slight precipitate	precipitation more marked	0	10
15	7·0	" "	" " "	3	13
10 and ammonia	8·3	" "	almost solid	36	40
Na_2SO_4					
0	7·0	clear	opalescent and slight deposit	0	10
5	7·0	slight precipitate	slight precipitation	2	10·6
10	7·0	" "	precipitation more marked	2	10
15	7·0	thick "	thick precipitate	15	—
10 and ammonia	8·3	slight "	" "	33	38
$ZnSO_4 7H_2O$					
0	7·4	clear	opalescent	0	17·7
15	more acid than p_H 4·5	thick precipitate	almost solid	protein completely precipitated during the heating	
20	"	" "	" "	" "	" "
$(NH_4)_2SO_4$					
0	6·8	thin suspension	thin suspension	0	—
30 saturation	6·8	"	precipitation increased	10	—
30 "	7·1	"	" "	12	—
30 "	7·6	"	" "	20	—
30 and 0·3 cresylic acid	7·2	"	" "	30	—
30 and ammonia	8·5	"	" "	44	—

In those cases where the sulphates of ammonium, potassium, magnesium or sodium had been used, the determination of the refractive indices of the heated and unheated sera of which the [H˙] lay between P_H 5·5 and 7·6, showed that the increased precipitation was due to an agglutination of colloidal particles of suspensoid protein into a suspension which ultimately settled as a perceptible precipitate. During the heating there had been no conversion of protein from the emulsoid to the suspensoid condition beyond that experienced in the control sera.

Where the reaction of the plasma had been more alkaline than P_H 8·0 the consistency of those sera containing sulphates in the proportions given in the Table, was invariably changed to that of a thick suspension; in the control experiments with plasma adjusted to the same [H˙] the heated liquids remained clear. In these cases the determination of the refractive indices of the sera containing sulphates showed that, during the heating, there had been an actual conversion of the emulsoid protein into a suspension. In the control experiments the changes induced had been insufficient to effect this conversion; the extent of the changes was detected by an estimation of the increased precipitability of the proteins.

From the data furnished with regard to the increased precipitability of the serum proteins by ammonium sulphate it will be seen that, except at concentrations of the electrolyte nearing those required for the precipitation of one or other of the serum proteins, the extent of the heat-denaturation was not directly affected by the addition of those sulphates which caused no appreciable change in the [H˙] of the plasma.

Thus, the extent of the heat-denaturation induced in plasma containing the sulphates of ammonium, potassium, magnesium or sodium, was practically the same as in the control experiments and was obviously regulated by the [H˙] of the plasma.

The addition of 15 and 25 % of zinc sulphate to the plasma changed the [H˙] from a value P_H 7·4 to values more acid than P_H 4·5. As was to be expected, there was complete precipitation of the serum proteins during the heating of plasma to which this sulphate had been added.

It is interesting to note that in the case of sera more alkaline than P_H 8·0 the increased precipitation of protein from the heated sera was approximately a measure of the extent of the heat-denaturation induced; the latter was in no way increased beyond that of the control sera adjusted to the same [H˙]. The addition of sulphates to these alkaline sera had thus induced a precipitation of heat-denaturated protein similar to that observed during the heating of acid sera, or of sera to which either phenol and its homologues, or ether or chloroform had been added.

The results so far obtained show that while the addition of sulphates to the plasma does not directly affect the extent of the heat-denaturation of the serum proteins, it considerably improves the aggregation of suspensoid particles of protein into a filterable form. Moreover, in alkaline sera showing a sufficiently high denaturation, they induce the precipitation of the heat-denaturated protein which would otherwise have remained in the disperse phase.

(*b*) *The increased production of proteins insoluble in brine during the heating of sera containing sulphates.*

Samples of the unheated and heated sera of which the reaction lay between P_H 6·6 and 7·6 were filtered. To a fixed volume of each was added twice the volume of a saturated solution of salt and sufficient solid salt to ensure saturation. From comparative tests it was seen that, in all cases, the amount of "brine-insoluble" protein was considerably increased during the heating of the sera; the increase was more marked in those sera containing sulphates than in the controls with the original plasma.

In the preceding sections of the paper it has been shown that the addition of acid or of trikresol to the plasma increased the production of "salt-insoluble" protein during the heating of the plasma: under certain conditions, the "salting out" of the thus denaturated proteins ceased to be of a reversible nature.

It has been established that sulphates also favour an increased precipitation of heat-denaturated protein by salt. In this case, however, the following evidence shows that in sera of which the reaction was of the order of P_H 7·8, the increased salting out of the denaturated proteins, presumably the result of an electrical charging of the protein molecules by the sulphions, can be reversed, to a certain extent, by sufficiently reducing the active mass of the latter.

A precipitate of heat-denaturated pseudoglobulin, which in the control sera was "brine-soluble," but which had been rendered insoluble in brine by the presence of sulphates, was suspended in a volume of brine twice that of the original serum taken for concentration. After several weeks' extraction it was found that not more than a trace of the protein had been redissolved.

The volume of the brine was then increased to eight times that of the original serum. After a week the amount of protein in solution was not visibly improved; after three weeks there was an appreciable improvement: by the end of three months a considerable amount had been dissolved, although, even after five months, the recovery was not complete.

The same phenomena are exhibited by the proteins precipitated by the addition of ammonium sulphate in excess of that required to precipitate "salt-soluble" heat-denaturated pseudoglobulin from solution. The ease with which the heat-denaturated pseudoglobulin, thus precipitated, is redissolved in brine depends partly on the extent to which the protein has been denaturated during the heating, and partly on the percentage of sulphate present. Thus, in a case under investigation, while the denaturated pseudoglobulin and antitoxin attached thereto precipitated by the addition of ammonium sulphate to the extent of 30 % of saturation, was readily dissolved by brine, that precipitated from the same solution by the addition of ammonium sulphate in greater amounts was not completely redissolved after six months' extraction with a large volume of brine. Moreover, it was found that, as the heat-denaturation was increased, so the reversibility of this precipitation was either diminished or delayed. Experimental work is in progress to settle this point.

(c) *The percentage removal of the serum proteins and the degree of concentration experienced during the concentration of sera containing sulphates.*

The data with regard to the percentage removal of the serum proteins and the increase in potency obtained during the process of concentration have been recorded in Table IV. A study of the Table shows that in those cases in which the percentage removal of the serum proteins had been greater than 50 % there were appreciable losses of antitoxin. Without doubt the losses were due to the increased insolubility of heat-denaturated protein and associated antitoxin in brine. For the recovery of the antitoxin thus precipitated it was necessary to submit the precipitates to a prolonged extraction in a large volume of brine.

In all cases the end products were more deeply coloured and, generally speaking, neither the percentage removal of the serum proteins nor the degree of concentration experienced was in any way better than that obtained in the preceding sections.

It was subsequently shown that the failure of the sulphates to improve the degree of concentration, in spite of the increase in "salt-insoluble" proteins which they favour, was due to the increased precipitation of albumin at the stage at which pseudoglobulin and antitoxin are precipitated.

Table IV.

Showing the results obtained from the concentration of sera by the direct treatment of the sera, heat-denaturated in presence of sulphates, with brine.

Electrolyte added to the plasma previous to its being heated	P_H of the plasma	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage removal of the total proteins of the original serum induced during the process of concentration	Percentage of the total antitoxic units of the original serum appearing in the residues from the dialysis of the protein precipitated by the addition of acetic acid to the filtered brined sera	Increased potency per cc. shown by the end product, calculated for purposes of comparison on the basis that they contain 20 °/o of protein
Controls —	7·0	10	27	—	—
—	7·4	18	35	—	—
—	7·6	28	48	> 90	4
$(NH_4)_2SO_4$ (30 % of saturation)	6·8	10	35	> 85	3·5
do.	7·2	10	35	> 85	3·4
do	7·4	20	48	88	4
do.	8·3	30	51·8	70	4
$MgSO_47H_2O$ (30 %)	7·2	13·5	60	70	5·9
Na_2SO_4 (10 %) and 0·3 % cresylic acid	7·6	40	57	70	5
Na_2SO_4 (7 %)	7·4	18	42·6	—	—

IV. THE INVESTIGATION OF THE REASON FOR THE LOWER DEGREE OF CONCENTRATION OBTAINED FROM THE "SALTING OUT" OF THE HEATED SERA WITH SODIUM CHLORIDE THAN FROM THE USE OF AMMONIUM SULPHATE.

Experimental work was next undertaken to ascertain the cause underlying the lower degree of concentration obtained by the direct treatment of the heated sera with salt as in sections I, II and III, than that which is usually obtained as a result of their fractional precipitation with ammonium sulphate.

The concentration of volumes of antitoxic sera, ranging from one to sixty litres, were carried out by the method under discussion and by the Homer [1916, 1918] method. A comparison was made between the respective end products as regards their protein content, the increased potency over that of the original serum and their antitoxic content. The results of the investigation have been embodied in Table V.

The use of ammonium sulphate as the salting out agent in the latter method resulted in end products which showed a potency per cc. about eight times that of the original serum; their protein content indicated that there had been a removal of about 75 % of the serum proteins during the process of concentration.

In the concentration of sera by the former method in which sodium chloride was used as the "salting out" agent the maximum increased

TABLE V. *A comparison between the composition of the end products obtained from the concentration of sera by the methods described in sections I, II and III of this paper and by the Homer* [1916, 1918] *method.*

Series No.	Substances added to the plasma previous to its being heated	P_H of the plasma previous to its being heated	Method of concentration adopted	Percentage of the total antitoxic units of the original plasma appearing in the end product	Increased potency per cc. shown by the end products, calculated for purposes of comparison on the basis that they contain 20 % of protein	Percentage removal of the serum proteins induced during the process of concentration	Percentage of *albumin* admixed with pseudoglobulin in the end product
Antitetanic sera							
57	—	7·6	Homer [1916]	> 90*	8·5	73·5	1·5
57	acetic acid	5·05	the method given in section I	88	5	55	21·0
57	ammonia	8·30	,, ,, ,,	88	5	54	22·0
87	ammonia	8·3	Homer [1918]	circ. 85*	8	78·5	2·0
80	0·30 % cresylic acid	7·2	,,	90	12	78·0	5·0
85	0·30 % ,,	7·1	,,	circ. 85*	7	78·3	5·0
85	$(NH_4)_2SO_4$ (30 % of saturation)	6·8	method given in section III	> 85*	3·5	35	36·5
85	,, ,, ,,	7·2	,, ,, ,,	> 82*	3·4	35	33·5
85	30 % $MgSO_4 7H_2O$	7·2	,, ,, ,,	70	5·9	60	38·0
85	10 % Na_2SO_4 and 0·30 % cresylic acid	7·2	,, ,, ,,	70	5	57	50·0
85	7·5 % Na_2SO_4	7·2	,, ,, ,,	—*	—	42·6	40·0
Antidiphtheritic sera							
55	0·30 % cresylic acid	7·6	Homer [1918]	circ. 88*	8	78·0	4·9
55	0·30 % ,,	7·6	method given in section II	88	4	51·0	35·5
55	ammonia	8·3	,, ,, ,,	88	4	46·3	23·5
55	0·30 % cresylic acid and ammonia	8·3	,, ,, ,,	88	4	46·8	35·5
73	$(NH_4)_2SO_4$ (30 % of saturation)	7·1	Homer [1916]	88	10·5	71·0	—
73	,, ,, ,,	7·1	method given in section III	> 90*	4	48·0	25
73	$(NH_4)_2SO_4$ (30 % and 0·30 % of cresylic acid	7·1	,, ,, ,,	70	4	51·8	50·3

* Not tested further because of the lack of experimental animals.

potency that was obtained without undue loss of antitoxin was of the order of five times; no improvement in this respect was effected by the addition of substances such as cresylic acid which are of proven value in the methods involving the use of ammonium sulphate.

It has been shown that cresylic acid materially improves the latter processes by inducing a greater removal of the serum proteins. However, in the method under consideration, the use of cresylic does not appreciably reduce the percentage of the original proteins appearing in the end product.

It was noticed that the end products from the brining of the heated sera were, without exception, more intensely coloured than those now obtained by the Banzhaf and the Homer methods. The colour was invariably most pronounced where the heat-denaturation has been conducted in presence of cresylic acid or sulphates.

It has been recently shown that in the Banzhaf and the Homer methods the colour of the end products depends to a large extent on the presence of heat-denaturated albumin and adsorbed colouring matter of the serum [Homer, 1917, 3, 5]. These observations together with those on the failure of cresylic acid and of sulphates to improve the percentage removal of the serum proteins during the process of concentration, led me to conclude that the end products from the direct brining of sera contain albumin in considerable amounts.

Experimental evidence in justification of this conclusion was furnished in the following way.

Albumin and pseudoglobulin were respectively precipitated from unheated plasma in the usual way. The precipitated proteins were twice redissolved and reprecipitated in order to free them as much as possible from admixture with the other protein entities of the serum. The precipitates after filtration were drained, pressed and dialysed in the usual way. The residues from dialysis were diluted so as to contain about 5 % of protein in solution.

It was found that there was no precipitation of protein from aqueous and from 0·85 % salt solutions of pseudoglobulin and of albumin respectively, of which the [H$^{\cdot}$] had been adjusted to values between P_H 7·0 and 3·0.

After the addition of electrolytes to the solutions, the precipitation of protein was readily induced at a [H$^{\cdot}$] between P_H 4 and 5. Thus, the solution of pseudoglobulin saturated with salt began to show slight precipitation on the addition of 0·05 % of glacial acetic acid (circ. P_H 5·5): the precipitation was complete after the addition of 0·30 % of the acid (circ. P_H 4·7). The solution of albumin, similarly treated, showed a slight precipitation on the addition of 0·15 % of glacial acetic acid (circ. P_H 5·0): while the precipitation was considerable at 0·3 % it was not complete until 0·5 % of glacial acetic acid had been added.

From these observations it follows that the protein precipitated by the addition of 0·25 % of glacial acetic acid to the filtered brined sera contains a considerable amount of albumin admixed with the pseudoglobulin.

Confirmatory evidence on this point was obtained by a gravimetric determination of the proportion of albumin to pseudoglobulin in the respective end products.

The results of the determinations have been included in Table V. They show that, where the [H˙] had been suitably adjusted to the desired value, the proteins appearing in the end product from the concentration of sera by the Homer [1916, 1918] method consisted of about ninety-eight parts of pseudoglobulin to two parts of albumin: where the alternative treatment of the plasma had been adopted, viz., the addition of 0·3 % of cresylic acid to the plasma previous to its being heated, the proportion of albumin rose to 5 %.

The protein appearing in the end products from the methods involving the brining of the heated sera contained a relatively greater amount of albumin: which latter depended on the extent of the heat-denaturation and on the nature of the substances added to the serum previous to its being heated.

In those cases in which the heat-denaturation had been regulated by an adjustment of the [H˙] to values between P_H 5·0 and 8·7, 20 to 25 % of the protein appearing in the end product consisted of albumin. Where the denaturation had been induced in presence of cresylic acid or of sulphates the proportion of albumin was considerably increased, in some cases being as high as 50 %.

The evidence thus adduced shows that, by the direct treatment of the heated sera with salt and the subsequent precipitation of the brined extracts with acetic acid, the degree of concentration cannot be so great as that attained by the use of methods in which the separation of the albumin is more or less assured.

From a study of the data given in Table V it will be seen that the best results were obtained from the brining of those sera in which, previous to their being heated, the reaction had been adjusted to about P_H 8·3.

The addition of electrolytes or of cresylic acid to the plasma adjusted in this way was of no material advantage, for while they induced an increased precipitability of heat-denaturated pseudoglobulin, the advantage thus gained was more than counterbalanced by the increased precipitability of heat-denaturated albumin.

In fact, for this reason, the addition of cresylic acid or of sulphates sometimes induced a lower degree of concentration than would otherwise have been obtained. Thus in experiments (not recorded in the Tables) where a high percentage removal of the serum proteins had been induced, the end products showed a potency of only 2–3 times that of the original serum: they also contained less than 50 % of the antitoxin taken for concentration.

The low degree of concentration was found to be due to the high percentage admixture of denaturated albumin with pseudoglobulin in the end product; the high percentage loss of antitoxin was due to its precipitation with the "brine-insoluble" heat-denaturated pseudoglobulin.

SUMMARY.

1. The concentration of antitoxic sera can be successfully conducted by a regulation of the heat-denaturation of the serum proteins followed by a direct treatment of the heated sera with brine and salt.

2. The end products thus obtained were clear and readily filterable, as they were not liable to contain the colloidal suspension of heat-denaturated protein which is so often found in those resulting from methods involving the use of ammonium sulphate.

3. Where the heat-denaturation had been of the order of 30 to 40 %, the use of this method led to the removal of about 50 % of the serum proteins. The potency of the end products was about five times that of the original serum.

4. The degree of concentration was not improved by a further increase in the extent of the heat-denaturation or by the addition of substances such as cresylic acid or sulphates to the serum previous to its being heated.

It is a matter of experience that the factors which increased the insolubility of the heat-denaturated pseudoglobulin in brine also increased the precipitation of heat-denaturated albumin at the stage at which the pseudoglobulin and antitoxin are precipitated.

5. In all cases the end products were more deeply coloured and contained a greater percentage of albumin than is found in those now obtained by the use of recent modifications of the Banzhaf [1913] and the Homer [1916] methods.

6. Under some conditions the heat-denaturated proteins precipitated

during the heating of sera containing cresylic acid or of which the reaction is more acid than P_H 5·0, do not redissolve in brine, even on long standing.

On the other hand there is evidence that the proteins precipitated during the heating of sera containing electrolytes can be redissolved by the prolonged treatment of the precipitates with a large volume of brine.

7. For the successful concentration of sera, the precipitation of those proteins to which the antitoxin is attached must not be changed from a reversible to a non-reversible type of reaction. In the latter case, even though the animal tests show that there has been no inactivation of the antitoxin attached to the insoluble particles of protein, from the point of view of the concentration of serum for clinical use this antitoxin is hopelessly lost until a method of dealing with the insoluble precipitates is forthcoming.

8. The method under discussion has not furnished end products showing so great a percentage removal of the serum proteins nor a degree of concentration so high as that now obtained in these laboratories by the Banzhaf and the Homer methods. Nevertheless the description of the method with sodium chloride may be of value to those engaged on problems connected with the routine concentration of sera, for, should untoward circumstances arise to prevent the concentration of sera by the methods now in use, there is hereby furnished an alternative method of proven practicability.

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XVIII. ON THE BIOCHEMICAL MECHANISM OF GROWTH. THE EFFECT OF SODIUM AND CALCIUM IONS ON THE GROWTH OF A TRANSPLANTABLE MOUSE CARCINOMA.

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(From the Laboratories of the Imperial Cancer Research Fund.)

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This paper contains an account of experiments in which rapidly growing mammalian cells, namely the cells of a transplantable mouse carcinoma, were exposed in vitro to the action of equimolecular solutions of sodium chloride and calcium chloride with the object of studying the resultant effect on their rate of growth.

The experimental details were as follows. A rapidly growing tumour strain—No. 63 of the Imperial Cancer Research Fund—was selected, which, when made into an emulsion and transplanted in measured doses with a syringe, grows evenly and in a high percentage (90–100 %) of the inoculated animals. A tumour, after being freed as much as possible from all necrotic tissue, was made with scissors into a fine emulsion. If one tumour did not yield sufficient material two or three tumours from the same generation were used and made into one emulsion. One portion of the tumour emulsion was transplanted directly into mice by means of the transplanting syringe in doses of 0·02 cc. This series served as a control. Approximately equal portions of the emulsion, amounting to about 0·5 cc., were transferred by means of the transplanting syringe to two centrifugal tubes. These tubes had a capacity of about 25 cc. and could be closed by means of a rubber washer and a metal screw cap. Equal volumes (about 12 cc.) of sterile $M/7{\cdot}5$ solutions of sodium chloride and calcium chloride in tap water were added to the two centrifuge tubes, so that the latter were about half filled. The tubes with the tumour cells suspended in the two salt solutions were then shaken gently in a shaking machine at room temperature for periods varying in different experiments from 70 minutes to 110 minutes. Since the tubes were filled to only half their capacity an aeration of the solution was maintained throughout this period. The suspensions were then centrifuged at a moderate speed for about three minutes, and the supernatant fluids, which contained a certain amount of protein, derived presumably from plasma and some necrotic tissue which had remained, decanted. The two masses of cells which had collected at the bottom of the two tubes were shaken out into two glass capsules and cut up again with scissors into an emulsion. The two emulsions were then transplanted by means of the transplanting syringe in doses of 0·02 cc. into two batches of mice. Aseptic conditions were observed throughout the experiment. The subsequent growth was then charted in the usual way in weekly intervals.

The experiments gave a uniform result in showing a distinct inhibition of the growth of those cells which had been treated with calcium chloride. This inhibition manifests itself both in a diminution of the number of positive inoculations and also in a lessened rate of growth of those tumours which had taken. This latter effect is only transient, as will be discussed presently. The cells which had been treated with sodium chloride gave the same high percentage of positive inoculations as the control batch and grew almost as rapidly as the control.

This result naturally raised the further question whether the inhibitory effect of calcium ions could be antagonised by sodium ions. This was tested by adding to the three series of inoculations used in the experiment which has just been described, a fourth series of inoculations in which the cells, after having been suspended in a calcium chloride solution, were further treated with a sodium chloride solution. This experiment was carried out in the same manner as the experiment detailed above, except that three centrifuge tubes were charged with equal portions of a tumour emulsion. Two of these tubes received a $M/7{\cdot}5$ $CaCl_2$ solution. After shaking these three suspensions for equal periods, centrifuging and decanting, the cells contained in one of the two $CaCl_2$ tubes were transplanted at once, as well as the cells which had been treated with sodium chloride solution. To the cells in the second $CaCl_2$ tube fresh $M/7{\cdot}5$ NaCl solution was added, in which they were suspended and shaken for a period of time approximately equal to that during which they had been exposed to the $CaCl_2$ solution. They were then centrifuged, made into an emulsion with scissors and transplanted in equal doses of 0·02 cc. The cells which had been treated in this way took again in 100 % of cases and showed a rate of growth almost, although not quite, as high as that of the cells subjected to the influence of the Na ions alone. This removal of the inhibition induced by Ca ions is all the more striking when one takes into consideration that the experimental manipulations and more especially the length of time during which the cells are being kept outside the body, are in themselves conditions not favourable to their subsequent growth. These experiments establish therefore clearly the existence of an antagonism between Na and Ca ions with reference to growth.

Fig. 1 which reproduces the chart of such an experiment illustrates the effects produced. The first column (mice Nos. 1–6) represents the growth of the control series, the second column (mice Nos. 7–14) the growth of cells treated with NaCl solution for 75 minutes, the third column (mice Nos. 15–24) the growth of cells treated with $CaCl_2$ solution for the same length of time, and the last column (mice Nos. 25–34) the growth of cells treated first with $CaCl_2$ solution for 75 minutes and then with NaCl solution for 45 minutes.

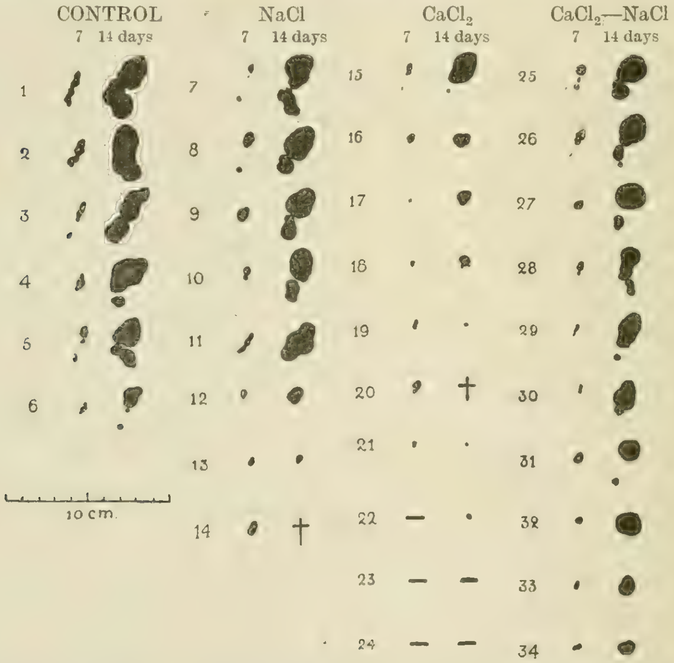

Fig. 1. Experiment 63/170 B. Chart showing size of tumours in two weekly intervals after transplantation. † indicates death of the animal from intercurrent disease; — indicates complete absence of tumour growth. For details of experiment see text.

In this experiment all the animals were killed after the second charting had been made and the tumours weighed. The results are given in Table I.

Incidentally the demonstration of this antagonism disposes of the possibility of attributing the diminished growth on the part of the cells treated with Ca ions to a merely lethal effect which kills off a number of cells and thus greatly reduces the number of living cells introduced with each dose.

TABLE I.

	Control		Na ions		Ca ions		Na ions after Ca ions	
	No of mouse	Wt of tumour in g.	No. of mouse	Wt of tumour in g.	No. of mouse	Wt of tumour in g.	No. of mouse	Wt of tumour in g
	1	3·7	7	1·9	15	1·1	25	2·4
	2	3·8	8	1·9	16	0·7	26	1·5
	3	2·7	9	1·9	17	0·3	27	1·6
	4	2·8	10	2·0	18	0·3	28	1·3
	5	2·0	11	1·8	19	below 0·1	29	1·1
	6	1·2	12	0·6	20	†	30	0·8
	—	—	13	0·2	21	below 0·1	31	0·9
	—	—	14	†	22	0·2	32	0·9
	—	—	—	—	23	—	33	0·7
	—	—	—	—	24	—	34	0·5
Total number of mice alive after 14 days	6		7		9		10	
Total weight of tumour growth in batch in g.	16·2		10·3		2·6		11·7	
Average weight of tumour per mouse in g.	2·7		1·5		0·3		1·2	

The inhibition of growth produced by Ca ions in these experiments is, as stated above, transient. If the tumours of the Ca strain are allowed to grow for more than two weeks they resume the rapid growth characteristic of the strain 63 from which they are derived. Indeed some of the tumours show a fairly rapid rate of growth already in the second week after inoculation. Similarly if the tumours of the Ca strain be reinoculated after two weeks without further experimental interference into fresh mice, these inoculations take again in 100 % of cases and grow as well as, if not better than, inoculations made from the control batch or from the Na strains.

It was of interest to determine what would happen if the inhibitory effect of Ca ions were applied repeatedly. Would the growing cells respond again and again to the action of Ca ions? Or would they get accustomed to it and fail to be inhibited in their growth? Or—a third possibility—would the inhibitory effect become more intense and perhaps permanent? In order to test these questions tumour cells were treated with a solution of calcium chloride as described above and inoculated. After two weeks some of the tumours obtained from these cells were made into an emulsion, of which one portion was inoculated at once while another portion was again subjected to the influence of Ca ions and then inoculated. This experiment was continued through five generations. The result eventually obtained in the fifth generation is shown in Fig. 2, which might represent equally well the results obtained in any of the preceding generations: in every case the control batch showed typical rapid growth in 100 % of inoculations and the Ca batch a transient inhibition, which did not appear to be either less or more distinct than the effect obtained when the treatment with $CaCl_2$ solution was applied for the first time. Nor was it possible to detect any histological difference between the tumours which had been subjected five times to the effect of Ca ions and the tumours of strain 63 obtained in the ordinary course of its propagation.

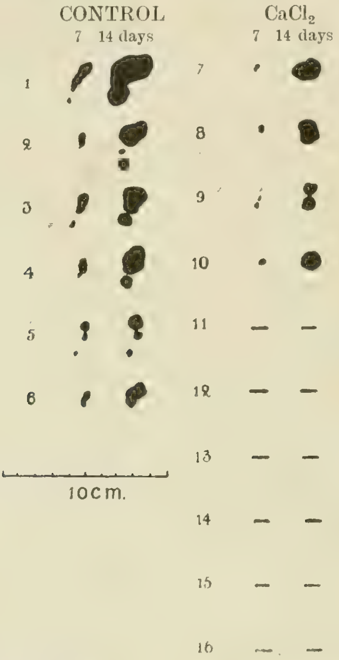

Fig. 2. Experiment 63/172 B. The material for transplantation had been taken from a strain which had been subjected at four successive transplantations to treatment with $CaCl_2$ solution. Part of the material has been transplanted directly ("control series"), another part has been treated again with $CaCl_2$ solution ("$CaCl_2$ series").

There is, however, a distinct difference recognisable both histologically and chemically, when the cells are examined immediately after the treatment with the various salt solutions. Histologically the protoplasm of the cells treated with $CaCl_2$ solution appears denser and less spongy than that of the cells treated with NaCl solution, or of the cells which have received no treatment at all. The dense protoplasm of the cells treated with $CaCl_2$ solution becomes looser again when these cells are treated subsequently with NaCl solution. These morphological differences have corresponding chemical differences in the water content of the cells, which is highest in the cells subjected to the influence of Na ions and lowest in the cells treated with $CaCl_2$ solution. In determining the water content the cells were suspended in the various salt solutions as has been described at the beginning of this paper. The suspensions were then centrifugalised, the mass of cells at the bottom of the tubes shaken out of the tubes and allowed to fall on blotting paper and the adherent fluid removed by touching the mass of cells gently with blotting paper. The cells were then transferred to weighing glasses and dried at 107° to constant weight. The results of two such experiments are given in the following table.

Water Content of Cells treated with:

Na ions	Ca ions	Na ions after Ca ions
85·4 %	83·8 %	86·8 %
85·1 %	84·0 %	85·2 %

These figures are probably slightly higher than the actual water content, since in removing the adherent water by means of blotting paper only the surface of the cake of cells can be dried and a certain amount of water which lies in the interstices between the cells always remains. A rough estimate of the amount of water thus retained is obtained when the cells are made into an emulsion and drawn up into the transplanting syringe for transplantation. The fluid then rises to the top of the emulsion. It is safe to say that the amount of this fluid is certainly not greater in the case of the cells treated with Na ions than with the cells treated with Ca ions, so that the comparative value of the estimations of the water content is not affected.

It is necessary to consider briefly to what extent the special experimental conditions may have contributed to the effect on growth observed in these experiments. There is firstly the fact that the cells treated with salt solutions were subjected to a greater number of mechanical manipulations and were kept for almost two hours at room temperature outside the body as compared with the control batch which was transplanted immediately. This factor operated evenly in the NaCl and $CaCl_2$ series, so that it cannot have contributed to the difference observed between these two series. Any influence which this factor may have had ought to have come out most clearly in the series in which the inhibitory effect of the Ca ions was antagonised by subsequent treatment with Na ions. It has been pointed out already that the restitution of the rate of growth in this series to the rate observed in the NaCl series adds to the conclusiveness with which the existence of an antagonism between the Na ions and Ca ions could be demonstrated. A second factor is the variation in the actual dose of cells introduced in the different series at each transplantation. After treatment with the various salt solutions the emulsions when taken up in the transplanting syringe were less coherent and looser than the emulsion used in the control series. Moreover, as already stated, they could not be freed entirely from a small amount of fluid which had remained between the cells after centrifugalisation. As transplantation proceeded and the emulsions were being pressed through the narrow transplanting needle they were compressed and became denser. Although the dosage by volume was kept constant at 0·02 cc., the actual dose of cells delivered each time must have been affected by this factor. In order to determine the degree of this variation the actual amounts delivered were weighed in a separate experiment. It was found that with the emulsion of the control series the amounts remained fairly even throughout a transplantation of fifteen doses, the actual amounts varying from 0·017 g. to 0·022 g. With the emulsion obtained after treatment with the various salt solutions, the first doses amounted to about 0·012 g. and gradually increased to 0·020 g. The NaCl series and the $CaCl_2$ series behaved in this respect in essentially the same manner. It is evident, therefore, that the differences in growth observed between the cells treated with the various salt solutions are not attributable to this factor. It may be partly responsible for the slightly more rapid growth of the control batch as compared with the series of cells treated with NaCl solution (either alone or after a previous treatment with $CaCl_2$ solution). For by varying, in a special experiment, the doses used for transplantation it was found that diminishing the dose of the control mice to 0·01 cc. slightly diminishes the initial growth during the first week but it does not diminish the percentage of positive inoculations, nor does an increase in the dose of cells treated with Ca ions to 0·03 cc. increase the percentage of successful inoculations. Moreover, although it slightly increases the initial growth of such cells, a Ca-series transplanted in doses of 0·03 cc. still shows a marked inhibition even if compared with a control series transplanted in doses of 0·01 cc.

General Discussion.

The inhibition of growth by Ca ions in its bearing on the general problem of growth. An interpretation of the inhibition of growth by exposing cells to the action of Na ions and Ca ions can be given on the basis of previous investigations on the biochemical mechanism of growth [Cramer, 1908, 1910, 1916; Cramer and Pringle, 1910, 1, 2; Cramer and Bullock, 1913]. In a series of observations an attempt had been made to determine the property or properties which enable rapidly growing cells to build up protoplasm more quickly than cells which exhibit a much slower rate of growth although living under identical nutritive conditions. The contrast is thus made, not between normal cells on the one hand and cancerous cells on the other, but between rapidly growing cells whether cancerous or normal (cells of testis, lymph nodes) and slowly growing cells whether cancerous or normal (cells of liver, kidney, muscle). By formulating the problem in this way it becomes restricted to one aspect of the general problem of growth and so kept separate from other aspects of this problem, such as the initiation of growth, or the extraneous conditions necessary for growth, diet for instance. These various aspects of the growth problem may or may not be interdependent. In the present state of our knowledge it has appeared to us advisable for the sake of clearness to treat them as if they were independent of each other. The material with which we have dealt consists of masses of cells differing markedly in their relative rates of growth while living under identical nutritive conditions. The question which we have tried to answer is: how do these various groups of cells maintain these differences in their relative rates of growth?

The answer to that question may be briefly summarised by stating that a rapidly growing cell works more economically in building up new living protoplasm than a slowly growing cell. It does so by using in the formation of new living protoplasm less of the complex organic colloids (especially proteins) and more water. The greater water content of rapidly growing cells is due to a greater imbibition on the part of the protoplasmic colloids.

If this conception of the problem of the biochemical mechanism of growth as a problem of imbibition is correct, it would follow that it should be possible to demonstrate a parallelism between differences in the rate of growth of different groups of cells and differences in the water content of their protoplasm. The existence of such a parallelism has been demonstrated in a previous paper [Cramer, 1916]. And conversely, it should be possible to alter the rate of growth by altering the imbibition of the protoplasmic colloids. This, we believe, is the explanation of the phenomenon recorded in the present paper.

The loss of water on the part of the tumour cells which has been demonstrated in this paper as resulting from exposure to a solution of calcium chloride recalls experiments [J. Loeb, 1899; Thunberg, 1909, 1910; Widmark, 1910, 1 and 2] in which a similar effect has been observed in the case of muscle cells. Reference may also be made to the inhibition by calcium salts of the imbibition produced in living tissues (muscle, eye, lens) and in non-living colloids (fibrin) by acids and alkalies [M. Fischer, 1908; Botazzi, 1911]. The antagonism between Na ions and Ca ions which has been demonstrated in the present paper with reference to growth holds good also for other activities of living protoplasm.

The inhibitory effect on growth produced by treatment with Ca ions conforms to the law enunciated by J. Loeb [1910] for the muscle cell. Loeb assumes that such a treatment alters within the cell the relative concentration of Na ions and Ca ions, which can be conveniently represented by the quotient C_{Na}/C_{Ca}. He states that a diminution of the value of this quotient produces an inhibitory effect. This holds good, as Loeb himself has shown, for the contraction of various forms of muscle. Thunberg has shown recently that treatment with $CaCl_2$ solution greatly diminishes the respiratory metabolism of the muscle cell.

This similarity between the process of growth and other manifestations of cell life suggests that growth also should be looked upon simply as a property inherent in living protoplasm in the same sense as metabolism, contractility and irritability. This is by no means a new conception. It is emphasised again in this connection, because the conclusions drawn by many recent workers on the growth problem frequently suggest or imply that growth is a property which can be added to or taken away from living protoplasm. Such expressions as cells "losing" or "acquiring" the power of growth suggest that growing cells contain or possess something which is absent from cells which are said to have "ceased to grow," and a similar conception is implied in the term "growth-substance." The subject is too big to be dealt with here in the course of a digression. It has been referred to because many difficulties which at present surround the problem of growth would, we believe, disappear if it were clearly recognised that there is no qualitative difference between cells with regard to growth: that all living cells possess normally the power of growth but in different degree. It is in some respects unfortunate that in most observations on growth the increase in weight (or length) of an entire, highly differentiated animal has been taken as a criterion of growth generally. This would imply as a premise that this increase in weight or length of an animal as a whole is the result of a process of growth which pervades the entire animal. But the increase in weight or length of such an animal is determined almost entirely by the increase in length of the skeleton. The absence of such an increase, which is often taken to be a criterion of an "arrest of growth" in its most general sense, is obviously not necessarily an indication of the absence of growth processes in the numerous other tissues of the animal. A histological inspection of the sexual organs and lymphoid tissues for instance of an adult animal leaves no doubt that processes of growth are actively proceeding in an animal in which the increase in length of the skeleton has terminated, as the result of the ossification of the epiphiseal cartilages in the long bones.

The inhibition of growth by Ca ions in its bearing on the growth of neoplasms. One of the first results of experimental cancer research was the demonstration of the fact that the growth of cancer cells, although continued and indeed unlimited, is not continuous [Bashford, Murray and Cramer, 1905; Bashford, Murray and Bowen, 1906]. The growth of a neoplasm shows definite fluctuations in the course of its experimental propagation: a certain strain of cancerous cells may at one time grow very rapidly, as indicated both by the percentage of successful inoculations and by the rapid increase in mass of the transplanted cells. Then, if propagation is continued, the percentage of successful inoculations diminishes suddenly, while those cells which have taken will show a very much slower rate of growth or may after an initial growth undergo spontaneous absorption. During such a period of depression of growth a tumour strain may even die out. If it is successfully carried over such a period, it will increase its rate of growth and maintain it for a time, until again a period of depression supervenes. This cyclical process, which is conditioned by factors inherent in the cell, indicates that the cancerous cell is subject to senescence and in that respect resembles the normal cell, but that it differs from the latter in being able to rejuvenate itself spontaneously and thus to escape death.

The significance of this phenomenon lies, apart from its theoretical importance, in the fact that it reveals a phase of diminished vitality in the life of the cancer cell which may serve as a guide in an attempt to find a rational therapeutic of cancer. Since cancer cells are subject to senescence, it is not unreasonable to expect that a knowledge of the factors on which this spontaneous process of sensecence depends may enable us to find conditions which might favour or maintain this process or even induce it. At present nothing is known about the factors which induce this process spontaneously. As to the nature of the process we have shown in a previous paper that the parallelism between rate of growth and degree of imbibition holds good for these fluctuations in the rate of growth in one and the same tumour strain. The phenomenon of the inhibition of growth recorded in this paper might be described as an experimental senescence of cancer cells, which it simulates also in showing the spontaneous rejuvenescence. It is obvious that the conditions by which senescence has been induced in vitro are crude compared with those which determine it in vivo. At present all that can be claimed for this experiment is that it affords an insight into the nature of the processes which lead to a temporary diminution of the vitality of cancerous cells.

SUMMARY AND CONCLUSIONS.

1. The effect of sodium and calcium ions on growth has been studied by suspending rapidly growing mammalian cells, namely the cells of a transplantable mouse carcinoma, in isosmotic solutions of sodium chloride and calcium chloride for one to two hours. Subsequent transplantation showed a very distinct inhibition of growth on the part of the cells which had been suspended in the calcium chloride solution. The growth of the cells which had been suspended in sodium chloride solution showed, in comparison with the control cells which had been transplanted without any further experimental interference, a slight diminution which is referable in part, if not entirely, to the additional experimental manipulations involved.

2. During the suspension in the calcium chloride solution the cells lose water and their protoplasm acquires a denser appearance.

3. Both the inhibition of growth and the loss of water produced by calcium ions can be antagonised by a subsequent suspension of the cells in sodium chloride solution.

4. The inhibitory effect on growth produced by calcium ions is transient.

5. An explanation of this effect has been given on the basis of previous observations in which it has been shown that the biochemical mechanism by which slowly growing and rapidly growing cells maintain the difference in their rate of growth is due to differences in the degree of imbibition of their protoplasmic colloids.

6. The inhibition of growth produced experimentally and the subsequent recovery therefrom present a similarity to the spontaneous fluctuations in growth which are exhibited normally by tumour cells in the course of their propagation.

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XIX. CREATINE EXCRETION IN RUMINANTS.

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Preliminary.

A considerable amount of evidence has accumulated to show that privation of carbohydrate or interference with its metabolism leads to an excretion of creatine in the urine. Thus Cathcart [1907] observed creatine in the urine of a starving subject, a like observation being made about the same time by Benedict [1907]. Cathcart [1909] later, found that in inanition the output of creatine is unaffected by feeding proteins or fat, but ceases on feeding carbohydrate. Mendel and Rose [1911, 1] caused the creatinuria of starvation in the rabbit to cease by giving dextrose or starch; protein or fat, singly or combined having no effect.

Excretion of creatine has also been shown in pathological conditions, natural or induced, that interfere with the metabolism of carbohydrate, e.g. diabetes mellitus [Krause, 1910], phloridzin poisoning [Taylor and Cathcart, 1910], destruction of carbohydrate in the tissues by injection of sodium selenite [Cathcart and Orr, 1914, 1] and in various conditions affecting the glycogenic function of the liver, e.g. hydrazine poisoning [Macadam, 1913].

Under the condition of privation of carbohydrate or its faulty metabolism, the acetone bodies that appear in the urine give rise to an error in the Folin method of quantitative analysis of creatinine and creatine, the former appearing less and the latter correspondingly greater than the true amounts [Greenwald, 1913; Graham and Poulton, 1914]. In most of the work referred to above this error was not guarded against and the amounts of creatine shown are doubtless exaggerated.

The creatinuria of starvation and of phloridzin poisoning have been re-investigated, the error of analysis due to acetone bodies being eliminated, and the original finding confirmed, though the creatine is less in amount and later in appearance than was formerly supposed [Cathcart and Orr, 1914, 2]. Further, the results obtained on rabbits by Mendel and Rose [1911, 1] are not affected by acetone bodies, which do not readily occur in rabbit's urine.

In all these researches the question turned upon the increased excretion of creatine in privation of carbohydrates in subjects that normally excreted no creatine or only small quantities. It was thought that further information might be obtained by experimenting in the opposite direction, i.e. giving a high carbohydrate diet to an animal that normally excretes creatine in large quantities and observing whether any quantitative relationship existed between the metabolism of carbohydrate and the creatine excretion. This method has the distinct advantage of getting rid of complications arising from the acetonuria of carbohydrate privation. To this end the following experiments were undertaken on ruminants (goats and sheep).

METHOD.

During each experiment the animal was put in a metabolic cage of such construction that the urine could be collected separately from the faeces. At the close of each experiment it was turned out to graze so that it might be in a normal condition at the commencement of the next period. Urine was collected in twenty-four hour periods and examined immediately at the close of the twenty-four hour period of collection. As no catheterisation was practised there was marked variation in the twenty-four hourly excretions. An approximation to the daily secretion is obtained by giving the average daily excretion of each separate period during which the conditions of experiment were constant. As the urine was examined on 142 days this method of presenting the results tends also to brevity and lucidity. The methods of analysis were: total nitrogen—Kjeldahl; creatine and creatinine—Folin.

I. PRELIMINARY EXPERIMENTS.

Creatine has been found in the urine of cattle [Lindsay, 1911]. The figures however are rather scanty and variable. Through the kindness of Dr Leonard Findlay several ruminants were rendered available and some preliminary work was done to ascertain to what extent creatine is normally excreted in ruminants. Table I gives the results obtained: the figures are the average daily amounts excreted for the periods stated.

TABLE I.

Creatine excretion in ruminants.

(Daily averages.)

Animal	Length of exper.	Tot. N g.	Creatinine g.	Creatine g.	Creatinine / Creatine ratio	Diet
He-goat, age 6 mon.	19 days	2·04	0·220	0·198	1·1	Bran, hay, turnips
Sheep, ewe, non-lactating	4 ,,	2·34	0·424	0·196	2·1	Hay and turnips
	4 ,,	?	0·446	0·397	1·1	Hay
Goat A, lactating, non-pregnant	7 ,,	7·61	0·620	0·457	1·4	Hay, oats, bran
	8 ,,	10·30	0·660	0·336	2·0	Hay, turnips, bran

In goat A, the total nitrogen figures include both milk and urine.

In the creatinine excretion of each animal there appeared a certain degree of uniformity not shown in the case of creatine which varied between 40 and 90 % of the amount of creatinine.

The urine was usually alkaline. To ensure that alkalinity of the urine had no influence in altering the proportion of creatinine and creatine several urines—dogs' and human—were treated with inorganic alkalis. No change was detected in the creatine or creatinine content. On nine days in the course of these experiments the urine was acid to litmus paper without any change in the proportion of creatinine or creatine being observed. The loss of creatinine which occurs in alkaline urines appears to be due not to the alkalinity *per se* but to organisms that flourish in an alkaline medium.

To ascertain the average creatine excretion under conditions as nearly normal as possible of goat B. in milk, that chosen for the following experiments, the animal was taken from grazing, put up in the metabolic cage and fed on turnips, oats and hay for five days. It was then let loose to graze for seven days and put up for a further period of nine days on the diet as before. The averages for fourteen days appear in Table II.

TABLE II.

Normal creatine excretion in goat B (lactating).

(Daily averages.)

Length of exper.	Tot. N urine + milk g.	Creatinine g.	Creatine g.	Creatinine / Creatine ratio	Milk cc.	Diet
14 days	5·04	0·85	0·31	2·7	79	Hay, turnips, oats

II. Influence of carbohydrate on creatine excretion.

Experiment I. This experiment was divided into four periods of five days each during which the diet was changed from a high protein and low carbohydrate to a low protein and high carbohydrate diet. The composition of the diets was as detailed below:

Period	Diet	Protein g.	N approx. (prot./6·25) g.	Fat g.	Carbohydrate g.
1	"Plasmon," 250 g. Bean meal 75 g. Turnip 200 g.	200	32	1·6	66
2	Potatoes, 1400 g.	14·98	2·4	1·4	224
3	Potatoes, aver. 1800 g.	19·26	3·1	1·8	288
4	Potatoes, 2200 g.	23·54	3·8	2·2	352

The composition of the potato diets is calculated from the average percentage of digestible nutrients given by Hendry [1913], viz. protein 1·07 %. carbohydrate 16·02 %. The figures given by Kellner [1909] vary as follows: protein 0·9 to 1·3 %; carbohydrate 12·5 to 24·6 %.

The animal was milked daily and the total nitrogen of the milk estimated, so that the combined nitrogen excretion of urine and milk might give some indication of the protein utilised in the periods of low protein diet. Table III gives the averages of the five day periods of the twenty consecutive days.

TABLE III.

Influence of carbohydrate on creatine excretion. Goat B (lactating).

(Daily averages.)

	Intake		Output					
Period	N approx. g.	Carbohydrate g.	Tot. N urine + milk g.	Creatinine g.	Creatine g.	Creatinine / Creatine ratio	Milk cc.	Change in wt. of goat kilos
1	32	66	28·11	0·91	1·13	0·8	219	loss 0·05
2	2·4	224	5·04	0·80	0·38	2·1	192	„ 2·2
3	3·1	288	4·08	0·98	0·23	4·2	176	„ 0·3
4	3·8	352	2·90	0·93	none	—	81	gain 0·3

Experiment II. In the foregoing experiment the flow of milk decreased from 219 to 81 cc. per day, so that an amount of material formerly drained off from the economy in the milk remained for consumption in the tissues. To obtain results uncomplicated by variations in milk flow this experiment (II) was delayed until lactation had ceased. The course of events during cessation of lactation is recorded in Experiment III. To eliminate any of the effects of the high protein diet being carried over to the subsequent period the animal was put upon a natural diet of turnips, hay and oats for an initial period of six days. It was then put upon a high carbohydrate diet of potatoes, which for the first period of three days was less than the animal's requirements. The diet was increased as follows:

Period	Length of Period	Diet		Protein g.	N approx. prot./6·25 g.	Fat g.	Carbohydrate g.
1	6 days	Turnips, hay, oats			*ad lib.*		
2	5 ,,	Potatoes,	960 g.	10·3	1·6	1	154
3	3 ,,	,,	2000 g.	21·4	3·4	2	320
4	3 ,,	,,	*ad lib.*	?	?	?	?

The results are given in Table IV.

Table IV.

Influence of carbohydrate on creatine excretion. Goat B (non-lactating).

(Daily averages.)

		Intake		Output				
Period	Length of period	N approx. g.	Carbohydrate g.	Tot. N g.	Creatinine g.	Creatine g.	Creatinine/Creatine ratio	Change in wt. of goat kilos
1	6 days	?	?	2·30	0·85	0·14	6	loss ·1
2	3 ,,	1·6	154	3·39	0·90	0·20	4·5	,, 1·6
3	3 ,,	3·4	320	3·14	0·91	0·18	5	,, ·1
4	3 ,,	3·4 +	320 +	2·35	0·92	none	—	,, ·3

The low creatine excretion in the initial period of six days is believed to be due to the fact that the animal had just ceased lactation, the last milk having been drawn off three days before the beginning of the experiment.

III. INFLUENCE OF CESSATION OF LACTATION ON CREATINE EXCRETION.

The following experiment intervenes in time between those recorded in Tables III and IV. The diet consisted of hay, oats and turnips. During the period, the animal was twice put to graze in the open, as a result of which the milk flow tended to return. While in the metabolic cage the flow steadily decreased. During the period the goat increased in weight from 32·7 to 35·4 kilos. The results are recorded in Table V.

RESULTS.

I. **CREATINE.** (1) *Normal Excretion.* In all the animals put under observation there occurs, except on the high carbohydrate diet, an excretion of relatively large quantities of creatine. In the various animals and on the various diets the amount fluctuates between rather wide limits. No constancy appears in the creatinine/creatine ratio, which in the preliminary experiments recorded in Table I varies between 2·1 and 1·1.

TABLE V.

Cessation of lactation, goat B.

(Daily averages.)

Length of period	Milk cc.	Tot. N urine + milk g.	Creatinine g.	Creatine g.	$\frac{\text{Creatinine}}{\text{Creatine}}$ ratio	Remarks
5 days	120	6·12	1·01	0·51	2	Milk flow constant
7 ,,	154		goat at grass			,, increasing
9 ,,	55	3·62	0·77	0·28	2·8	,, decreasing
10 ,,	11		goat at grass			,, increasing
7 ,,	6	5·33	1·02	0·26	3·9	,, decreasing
7 ,,	1	2·82	0·88	0·23	3·8	,, ,,
6 ,,	nil	2·30	0·85	0·14	6	,, ceased

(2) *Influence of Carbohydrates.* The highest creatine excretion—1·13 g. per day—occurs on the diet with the abnormally low carbohydrate content—66 g. per day. In both Tables III and IV as the carbohydrate content of the diet is increased, the creatine excretion decreases. The close relationship that exists is shown in Table VI.

TABLE VI

	Period	1	2	3	4
Table III	Carbohydrate in diet, g.	66	224	288	352
	Creatine excret., g.	1·13	0·38	0·32	nil
Table IV	Carbohydrate in diet, g.	?	154	320	*ad lib.*
	Creatine excret., g.	0·14	0·20	0·18	nil

The decrease is more rapid in Table III. This may be because the effects of the increased carbohydrate ingestion are augmented by the increased retention of material in the blood due to the reduction in the milk flow, which occurred during the course of the experiment.

(3) *Influence of Cessation of Lactation.* Table V shows a decreased creatine output accompanying the decreased milk flow. The two declines do not run parallel and the greatest decrease in the creatine occurs on the six days after the flow had completely ceased. During the period covered by Table V the animal increased in weight by 2·7 kilos.

(4) *Influence of Proteins.* In periods 2, 3 and 4 an increased nitrogen intake of—in g. per day 2·4, 3·1 and 3·8 coincides with a decreasing creatine output of 0·38, 0·23 and nil respectively: a like decreasing creatine output

occurs simultaneously with an increasing nitrogen intake in Table IV. On the other hand, the highest creatine excretion occurs on the highest protein intake, and in general in both Tables III and IV the decreased creatine excretion is accompanied by a decreased total nitrogen excretion.

In Table III on the fifteen days of periods 2, 3 and 4 the total nitrogen excreted amounts to 60·1 g. while the total nitrogen intake is 46·5 g.—a loss of 13·6 g., indicating a catabolism of about 85 g. of tissue protein. There is no indication of this endogenous protein catabolism producing any addition to the excretion of creatine, which during the period decreases to zero.

II. CREATININE. The excretion of creatinine shows remarkable uniformity, compared with the wide fluctuations shown by the excretion of total nitrogen and of creatine. Thus, in Table I, in the two periods of the ewe the amount, in g. per day, is 0·424 and 0·446 and in goat A 0·62 and 0·66. In goat B the highest is 1·02 and the lowest 0·77.

Taking the preliminary period of goat B, Table II, as the normal, we have 0·85 g. per day in an animal of 35 kilos, i.e. 24 mg. per kilo, a ratio somewhat similar to that found in dogs, and in man, suggesting a similar metabolism.

DISCUSSION OF RESULTS.

1. *Production of Creatine.* The fact that in a period of progressively increasing intake of protein of constant composition there occurs a decreasing creatine excretion (Tables III and IV) seems to indicate that the creatine, of that period at least, is not derived from the catabolism of exogenous protein. On the other hand the continuous excretion, in ruminants, of large quantities of creatine on a normal diet, as well as on a creatine-free high protein diet, points to a production of creatine from a source other than the catabolism of endogenous protein.

These results suggest that while quantities of creatine may appear as a consequence of the rapid disintegration of muscle tissue, or of the catabolism of certain exogenous proteins, these are merely irregular and adventitious increments to a constant creatine production in the tissues.

The results provide no means of estimating the amount produced. If, as is suggested below, creatine is utilised, the amount excreted would merely be the surplus of production over utilisation. In the fourteen days' period of goat B, Table II, the daily average on a diet of turnips, oats and hay is 0·31 g. On the lowest carbohydrate diet it is 1·13 g. while on the highest carbohydrate diet the excretion reaches zero. Provided there is no creatine of exogenous origin, there appears to be the possibility of the production and utilisation of over a gram per day.

On the whole the results would seem to lend support to the view that creatine is produced within the tissues in relatively large amounts, a conclusion arrived at by Benedict and Osterberg [1914], from results obtained on a dog under the influence of phloridzin.

2. *Fate of Creatine.* In so far as the creatine under discussion is being produced within the metabolic field, it is probably in a more favourable position for conversion to creatinine if such takes place, than is creatine given by the mouth or injected subcutaneously. The relatively uniform excretion of creatinine gives no indication of any conversion. If creatinine is derived from creatine the amount converted depends upon some constant metabolic process which is not affected by the amount offered for conversion.

Van Hoogenhuyze and Verploegh [1908] concluded that creatine is usually broken down in the liver. Noël Paton and Mackie [1912] ligatured the portal vein and its radicles in a duck and found no change in the creatine excretion. Towles and Voegtlin [1911] in a dog with an Eck's fistula found no change in the creatine or creatinine excretion. Dakin [1907] and Twort and Mellanby [1913] failed to discover any ferment in the liver capable of acting upon creatine. The influence of the liver in the metabolism of creatine is evidently indirect and possibly solely by virtue of its glycogenic function.

The question as to the fate of creatine cannot be regarded as settled by assuming either its destruction in the liver or its conversion to creatinine.

In the present experiments it has been shown that in an animal normally excreting creatine, the excretion decreases *pari passu* with the addition of carbohydrate to the diet, a result which is the complement of that arrived at by the workers referred to above, viz. the excretion of creatine in carbohydrate privation in an animal normally excreting no creatine. A close relationship therefore appears between the metabolism of carbohydrate and that of creatine. It can be stated at least, that increased carbohydrate ingestion and decreased creatine excretion are in some way causally connected.

Two possible views are considered. (1) Creatine is only produced in the absence of carbohydrate. (2) Creatine or its precursor is constantly being produced but is utilised in the metabolism of carbohydrates.

According to the first view, the excretion of creatine is an indication of the break-down of muscle tissue, and carbohydrate exerts its influence on the excretion by virtue of its presence preventing the necessity for this break-down. In all probability this source supplies at least a portion of the creatine excreted in starvation, in which the excretion of creatine is in some degree proportional to the break-down of tissue protein [Noel Paton, 1910]. As has been suggested above, however, the quantities of creatine excreted in ruminants cannot wholly be accounted for by the catabolism of endogenous protein, so that this view seemingly fails to cover all the facts as now presented.

The second view—the utilisation of creatine, a conclusion arrived at by Cathcart [1912], from the results of work done in this laboratory, and later suggested by Benedict and Osterberg [1914]—finds support in the fact that on the high carbohydrate and low protein diet, where there is evidence of a break-down of tissue protein and, presumably, the liberation of creatine, there appears in the urine no additional creatine, the excretion of which during the period decreases to zero.

If, as has been suggested, there occurs a constant production of creatine, the decrease in the excretion concomitant with the increased ingestion of carbohydrates points to a utilisation of creatine in the metabolism of carbohydrate.

If this view were correct one would expect to find, that, in cases of excessive consumption of carbohydrate, the creatine in the tissues would be utilised in excess of the production and the stock diminished. Myers and Fine [1913] record a decrease in the percentage of creatine in muscle from 0·520 to 0·482 on feeding on a pure carbohydrate diet. On the other hand, when carbohydrate is not presented for consumption there would be an accumulation and excretion of creatine. Demant [1879] observed an increase in the creatine content of muscle during starvation, an observation confirmed by modern methods by Mendel and Rose [1911, 2], while the excretion of creatine in inanition has been repeatedly observed, *vide supra*.

It may be, that creatine or whatever body represents it in the tissues serves as a connecting link securing the incorporation of the carbohydrate molecule within the protoplasm complex in some glucoside like combination, in which the catabolism of the carbohydrate moiety occurs.

CONCLUSIONS.

1. In ruminants creatine is normally excreted in relatively large quantities.
2. The amount of creatine excreted is in inverse proportion to the amount of carbohydrate in the diet.
3. Cessation of lactation causes a decrease in the amount of creatine excreted.

4. There is a source of creatine other than the catabolism of protein either exogenous or endogenous.

5. Arguments based on the results are advanced in support of the view, that creatine is formed in relatively large quantities, that it is utilised in the tissues and that its utilisation is intimately connected with the metabolism of carbohydrate.

This paper is an extract from a section of a thesis submitted for the M.D. degree of Glasgow University in May 1914.

The author desires to acknowledge his indebtedness to Professors Noël Paton and E. P. Cathcart for advice in connection with this research, and to the former for very helpful criticism in the preparation of this paper for publication.

Further research on the excretion of creatine on ruminants was being carried out in the summer of 1914. It is hoped to resume the work as soon as an opportunity is afforded.

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XX. AN INVESTIGATION OF THE METHODS EMPLOYED FOR COOKING VEGETABLES, WITH SPECIAL REFERENCE TO THE LOSSES INCURRED. PART I. DRIED LEGUMES.

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The value of dried legumes as articles of diet has long been known, but the present necessity for economising other foodstuffs has led to far more general recognition of the use and importance of these vegetables.

At the request of the Foods Committee of the Ministry of Munitions this investigation was undertaken with a view to obtaining some data as to the losses incurred and the time required for cooking dried vegetables under various conditions.

It was thought that from the information thus gained it would be possible to devise a convenient method of cooking dried legumes on a large scale which, whilst satisfactory in other respects, would minimise as far as possible the waste of food material due to losses in cooking and also reduce the time of cooking, thus effecting a saving in the consumption of fuel.

Some information on the subject of dried legumes is to be found in the bulletins issued by the United States Department of Agriculture [Abel, 1900, *Farmers' Bulletin*, 1903; Wait, 1907].

These publications discuss briefly the cooking of dried legumes but deal chiefly with their nutritive value and digestibility; their scope is therefore somewhat different from that of the present enquiry.

The varieties of dried legumes most commonly used in this country appear to be butter beans, haricot beans, and peas. As the butter beans were found to be rather more uniform in size and shape than the other varieties, most of the experiments were made with these and a few experiments were then made with haricot beans (white and brown) in order to see if similar results were obtained.

A study of the methods usually advocated for cooking dried legumes seemed to indicate that the subject could be dealt with best in the following sections:

I. The use of tap and distilled water for cooking beans and the effect of soaking previous to cooking.

II. The use of sodium bicarbonate in cooking beans.

III. The use of salt in cooking beans.

IV. The possibility of cooking beans by steaming.

V. Note on haricot beans and peas.

VI. Suggested methods of cooking dried vegetables.

I. THE EFFECT OF SOAKING BEANS PREVIOUS TO COOKING AND OF SOAKING AND COOKING IN DISTILLED AND TAP WATER.

Dried beans are usually soaked in water for at least eight hours previous to cooking. The beans so treated take up water, swell considerably and are generally supposed to cook more rapidly than those which have not been soaked previous to cooking.

The use of soft water in preference to hard water for soaking and cooking beans is also usually advocated since the presence of calcium salts in the water is stated to retard the softening of the beans and prolong the time of cooking[1].

A series of experiments was therefore made in order to compare the time of cooking and the loss in solid matter during cooking of beans both dry and previously soaked and to find how far these results were modified by using distilled water as compared with ordinary London tap water. (Temporary hardness = 12 parts per 100,000. Permanent hardness = 5 parts per 100,000.)

The dry beans were heated in a steam oven to constant weight and were found to contain 12 % of moisture which has to be allowed for in relation to losses incurred in cooking.

The experiments were carried out under conditions which reproduced as far as possible those of the ordinary kitchen. The method adopted for cooking the beans was as follows:

A. Beans cooked without previous soaking.

100 g. of beans were placed in a strainer and washed with cold water in order to remove any adhering particles of dirt. The beans were then placed in an enamel saucepan, 500 cc. of cold water were added, the saucepan was covered with a lid and the temperature of the water was raised to the boiling point and kept at this temperature until the beans were cooked. The time required for cooking was then recorded.

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[1] See Robert Hutchison, *Food and the Principles of Dietetics*, also general statements in cookery books.

B. Beans soaked in water previous to cooking.

The beans (100 g.) were washed with cold water as in *A* and were then covered with 250 cc. water and allowed to stand overnight (*i.e.* about 16 hours). The soaking water was strained off, measured and evaporated to dryness and the residue weighed in order to determine the loss in solid matter due to soaking. The beans were then placed in an enamel saucepan and covered with 500 cc. water less the volume of water taken up by the beans in soaking; this was found to be in each case about 100 cc. so that the volume added was about 400 cc. The cooking was then carried out as in *A*.

Cooked beans. The cooked beans were in each case separated as completely as possible from the cooking water by straining, allowed to cool at the room temperature and weighed when cold. This weighing gives the weight of the cooked beans in the condition in which they are ordinarily served. The appearance and taste of the beans were then examined and reported on.

Cooking water. The cooking water was evaporated to dryness and the residue weighed. This residue gives the total solid matter extracted from the beans in cooking together with the residue, if any, from the water used. A correction was made where necessary for such residue.

Since the dry uncooked beans used for the experiments were found to contain 12 % of moisture the residue from the cooking water represents the loss of solid matter on 88 g. of solids, from which figure the percentage loss, *i.e.* the loss on 100 g. of solids, was calculated. The time of cooking, weight of cooked beans and percentage of loss of solid matter in cooking as observed in a number of experiments both with tap and distilled water are given below.

TABLE I. (Tap water.)

A. 100 *g. beans cooked in tap water without previous soaking*

Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking
2 h. 10 m.	244	12·5
2 15	223	12·8
2 0	230	—
2 15	—	11·6
2 15	229	12·9
2 0	230	10·9
2 15	235	8·5
2 10	219	8·1
2 15	237	11·1
2 0	232	9·3
2 0	214	9·6
2 0	211	8·6
Average 2 h. 5 m.	220·5	10·5

B. 100 *g. beans soaked and cooked in tap water*

Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in soaking	Total percentage loss of solids
2 h. 0 m.	231	13·6	1·8	15·4
2 0	235	8·4	2·2	10·6
2 0	—	11·5	1·7	13·2
1 50	210	8·2	1·5	9·7
2 h. 0 m.	228	10·4	1·8	12·2

TABLE II. (Distilled water.)

A. 100 g. beans cooked in distilled water without previous soaking			*B. 100 g. beans soaked and cooked in distilled water*				
Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in cooking	Total percentage loss
1 h. 45 m.	242	—	1 h. 45 m.	231	12·8	2·2	15·0
1 45	218	13·7	1 45	235	9·4	2·2	11·6
1 45	213	11·6	1 45	—	10·7	1·7	12·4
1 45	229	10·7	1 55	208	8·6	—	
2 0	205	8·6					
Average							
1 h. 45 m.	218	11·1	1 h. 45 m.	224	10·4	2·0	12·6

Discussion of results of Tables I and II.

Time of cooking. It will be seen from the results given above that soaking previous to cooking does not reduce the time required for cooking to any very marked extent, but that the time required with distilled water is less than in the case of tap water.

Cooked beans. Soaking beans previous to cooking has practically no effect on the weight of the beans when cooked, the weight of cooked beans being in each case rather more than double that of the raw beans. The soaked beans were a better colour, *i.e.* whiter, and had rather a less pronounced flavour than those which were cooked without previous soaking. The beans cooked in tap water had a better flavour than those cooked in distilled water, which were rather tasteless.

Loss in solid matter. As regards the loss in solid matter during cooking the mean results show that the loss with tap and distilled water is practically the same. Taking into consideration the solid matter extracted by soaking, the total loss in the case of the soaked beans is greater than in the case of those cooked without previous soaking. The individual experiments show considerable variation in the amount of solid matter extracted during cooking; this no doubt is due mainly to the difficulty of determining the exact stage at which the beans are cooked.

The method usually adopted in the kitchen is to prod the beans with a fork and to stop the cooking when the beans have reached what is considered to be the required degree of softness. Since the experiments were to be carried out as far as possible under conditions similar to those of an ordinary kitchen the same method was adopted in the laboratory though it is obviously open to objection.

When the beans are tested the skin is pierced and this will tend to increase the amount of solid matter removed. This is especially the case when the cooking of the beans is nearing completion. The results are given in the order in which the experiments were carried out and it will be noted that the earlier experiments showed, on the whole, greater losses than the later ones.

This would be accounted for by the fact that at first some difficulty was experienced in deciding when the beans were cooked; they had to be tested several times and there was probably also a tendency towards overcooking; whereas in the later experiments more experience had been gained in judging the end point, the beans were not tested so frequently and the cooking was stopped before the beans began to disintegrate.

It might be thought that the time of cooking would indicate whether the beans had been overcooked or not, but this is not a sufficiently reliable check as owing to the difficulty of exactly regulating the gas supply the time required for cooking was found to vary slightly from day to day and it was noticed that if the cooking was not stopped when a certain stage was reached the beans began to break up rather rapidly and that in the case of peas this tendency was even more marked than in the case of beans.

In order to show the effect of overcooking on the loss in solid matter the beans in one experiment were purposely overcooked, the time allowed being 2 hrs. 25 mins.: the loss in solid matter was then found to be 16·6 % of the total solids.

It would of course be possible to employ a more uniform source of heat and it is hoped to make some experiments under such conditions later.

For the purpose of the present enquiry however it was thought best to adopt such methods as would ordinarily be employed in the kitchen. In dealing with other methods of cooking it was therefore decided that several experiments should be made in each case and an average then taken, and a control experiment with tap water made from time to time. It was hoped that in this way the errors dealt with above might be to some extent eliminated.

Nature of residue. The residues obtained from the soaking and cooking waters have not yet been fully examined and the discussion of their exact nature must be left until some further investigations of the subject have been made.

Residue from soaking water. The residue from the soaking water is dark brown in colour, difficult to dry and if exposed to the air rapidly takes up water and becomes syrupy. The odour and taste is somewhat similar to that of a meat extract. The residue from dry beans soaked in tap water was found to contain 2·3 % of nitrogen probably in the form of extractives and the mineral matter extracted by soaking was 1·54 % of the total mineral matter present in the beans.

Residue from cooking water. The residue from the cooking water is lighter in colour than that from the soaking water and is less deliquescent. The taste and odour are similar but less pronounced.

The residue from dry beans cooked in tap water contains 2·4 % nitrogen corresponding to a loss of 7·5 % of the total protein matter present in the beans and the mineral matter extracted by the cooking is 36·8 % of the total mineral matter present in the beans.

A few experiments were also made to see if there was any advantage to be gained by (1) cooking the beans in the water in which they had been soaked, (2) cooking the beans by the addition of boiling instead of cold water. The results obtained however were very similar to those given above.

In (1) the residue from the cooking water was equal to the combined residue obtained from the soaking and cooking waters in the previous experiments.

In (2) allowing for the time required for bringing the water to the boil the time required for cooking was the same as in the above experiments and the solid matter extracted by cooking was about the same.

II. THE USE OF SODIUM BICARBONATE IN COOKING DRIED BEANS.

Sodium bicarbonate and also washing soda are sometimes added in small quantities to the water in which dried legumes are to be cooked or if they are soaked previous to cooking it may be added to the soaking water.

It is usually stated that this treatment softens the water and so tends to reduce the time of cooking[1].

Although the degree of hardness of the water used for cooking must be taken into account in determining the amount of sodium bicarbonate which should be added it would appear from the experiments now to be described that the alkali has a more specific action than can be accounted for by the above explanation.

In this connection it may be mentioned that Snyder [*Minnesota Station Bulletin*] states as a matter of common observation that when beans are properly prepared with a small amount of soda and salt there is less formation of gas in the intestines during digestion. A series of experiments was therefore made in order to compare the times of cooking and the loss in solid matter when beans were cooked (*A*) with the addition of varying amounts of sodium bicarbonate to the cooking water, (*B*) after soaking in water to which varying amounts of sodium bicarbonate had been added.

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[1] See Hutchison, *Food and the Principles of Dietetics*, also general statements in cookery books.

The quantities used, the method of cooking and the subsequent treatment of the cooked beans and cooking water were the same as in the previous experiments.

A. The addition of sodium bicarbonate to the cooking water.

Beans were cooked, without previous soaking, in tap water to which a weighed amount of sodium bicarbonate had been added. The quantity of alkali left in the cooking water at the end of the experiment was estimated and the residue corrected by a corresponding amount. The mean results of these experiments are given below:

TABLE III.

Beans (100 *g.*) *cooked in tap water containing sodium bicarbonate.*

(Average results.)

Parts of sodium bicarbonate per 100 parts water	Time of cooking	Weight of cooked beans	Percentage-loss of solids in cooking
0·05	2 h. 0 m.	221	10·3
0·1	1 50	216	11·3
0·25	1 45	225	14·3
0·5	1 15	240	22·3
1·0	1 0	190	27·3

The results are also shown graphically below. The logarithms of the relative concentrations of sodium bicarbonate used have been plotted instead of the actual concentrations so that equal changes in the concentration may be represented by equal lengths of ordinate.

Graph showing the Loss of Solid Matter compared with the Concentration of Sodium Bicarbonate used.

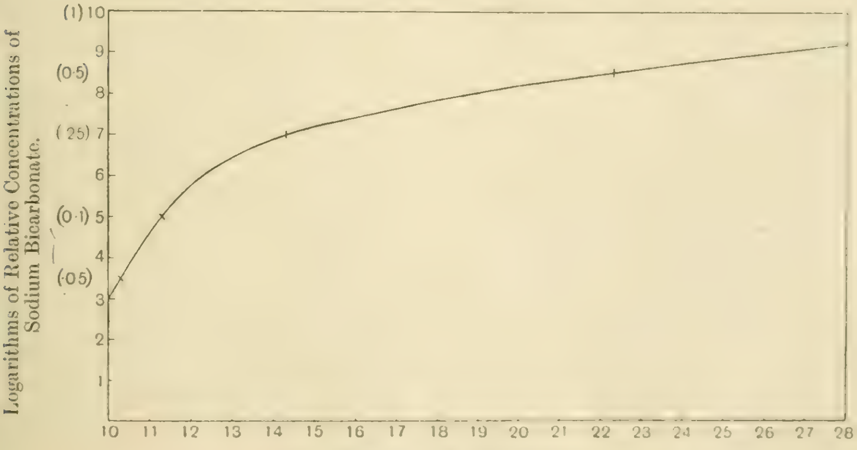

Fig. 1.

Discussion of the results of Table III.

The effect of the addition of sodium bicarbonate both on the time of cooking and on the loss in solid matter is somewhat striking.

The high percentage loss of solids obtained when the sodium bicarbonate added is as high as 0·5 1·0 % is attributable to the fact that under these conditions the beans soften and disintegrate rapidly, hence a large proportion of solid matter passes into the cooking water, and the latter becomes very turbid. The beans become distinctly yellow in colour, are slimy to the touch, and practically uneatable.

Similar effects, though in a much less marked degree, were also observed when 0·25 % sodium bicarbonate was used.

The most satisfactory results (for the particular tap water used) were obtained when 0·1 % of sodium bicarbonate was used.

As shown in the above results, the time of cooking was then the same as the time required with distilled water. The loss of solid matter was only slightly greater than in the case of tap water (Table I), and was slightly less than the total loss observed when the beans were soaked and then cooked in tap water.

There is no advantage to be gained by adding quantities of sodium bicarbonate less than 0·1 %, since the time required with the addition of 0·05 % is the same as when untreated tap water is used (Table I).

More information is needed before any complete explanation of the action of sodium bicarbonate can be attempted, but the solubility of the legumin of the beans in alkali must play an important part, and it is interesting to note that Osborne [1909], in dealing with vegetable proteins, states that "the amount of protein extracted by alkalies is usually greater than that extracted by neutral saline solutions or by water, in fact in many cases it is very much greater. Sufficient attention has not yet been directed to the cause of this difference and for this reason our present knowledge of the character of the total protein constituents of the greater number of seeds which have been studied is incomplete."

B. The addition of sodium bicarbonate to the soaking water.

Beans were soaked overnight in tap water containing varying amounts of sodium bicarbonate. The soaking water was strained off, measured and evaporated to dryness, and the residue weighed. An estimation was made of the quantity of sodium bicarbonate left in the soaking water which was then deducted from the total residue obtained.

The amount of sodium bicarbonate absorbed by the beans during soaking was found, for each concentration of the alkali, to be proportional to the volume of water absorbed which was about 100 cc. in each case.

The average results obtained are shown in Table IV.

TABLE IV.

Beans (100 *g.*) *soaked overnight* (*about* 16 *hours*) *in tap water containing sodium bicarbonate.*

(Average results.)

Parts of sodium bicarbonate per 100 parts water	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in soaking	Total percentage loss
0·25	1 h. 15 m.	222	10·6	1·8	11·4
0·5	1 15	214	9·2	1·5	10·8
1·0	1 0	211	8·7	1·5	10·8
2·0	1 0	212	13·1	1·5	14·6

TABLE V.

Beans (100 *g.*) *soaked for four hours in water containing sodium bicarbonate.*

(Average results.)

Parts of sodium bicarbonate per 100 parts water	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in soaking	Total percentage loss
1·0	1 h. 30 m.	222	10·8	0·2	11·0
2·0	1 30	234	12·0	0·2	12·2

Discussion of Tables IV and V.

The time required for cooking the beans after soaking in water containing sodium bicarbonate is considerably less than when either tap or distilled water is used (Tables I and II).

The reduction in time thus effected is fully as great as that observed when sodium bicarbonate up to 1 % is added to the cooking water and the reduction in the former case is not accompanied by the objectionable features noted in the case of the latter. Further experiments show that when 2 % of sodium bicarbonate is used the time of cooking is the same as in the case of 1 %, but the loss in solid matter is greater. The beans also were yellowish in colour and exhibited, though to a less degree, other objectionable features noticed in the case of beans cooked in water containing over 0·2 % of sodium bicarbonate (Table III).

The addition of sodium bicarbonate to the soaking water appears to diminish the amount of solid matter extracted from the beans by soaking, and it was also noted that these residues were lighter in colour than those obtained from tap water (see Section I).

This effect is somewhat curious, but the whole question of the action of sodium bicarbonate needs to be more fully investigated under more carefully controlled conditions and it is hoped that such an investigation may be made at a later date.

Table V shows the average results obtained when the beans are soaked for a shorter period, namely four hours, since it was thought that in some instances this might prove to be a more convenient method. The results show that the reduction in time of cooking thus effected is not so great as when the beans are soaked for a longer period, but the time required is appreciably less than that required with tap or distilled water (Tables I and II).

III. The use of salt in cooking dried legumes.

Vegetables are usually cooked in water containing salt with a view to improving their flavour, but in the case of dried legumes the statement is frequently made in cookery books that salt should not be added to the cooking water, as it tends to harden the skins and prolong the time of cooking and that this difficulty may be avoided by delaying the addition of the salt until the cooking is nearing completion.

It therefore seemed advisable to make a few experiments in order to compare the time of cooking and loss in solid matter when beans were cooked in water containing varying amounts of salt.

The method of cooking and subsequent treatment of the cooked beans and cooking water were the same as in the previous experiments. An estimation was made of the quantity of salt left in the cooking water at the end of the experiment, which was then deducted from the total residue.

The average results obtained when unsoaked beans were cooked in tap water containing salt are given below:

Table VI.

Beans (100 *g.*) *cooked in tap water containing salt.*

(Average results.)

Parts of salt per 100 parts of water	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking
0·25	2 h. 0 m.	215	9·2
0·5	2 0	212	8·7
1·0	2 15	216	8·3

Discussion of results of Table VI.

These results show that contrary to the usually accepted statement, the addition of salt, in small quantities, to the cooking water, does not appreciably increase the time of cooking.

The loss in solid matter tends to decrease with the addition of salt (compare Table I), and the flavour is considerably improved.

The most satisfactory result was obtained when 0·25 % was added, the flavour of common salt being considered rather too pronounced when larger quantities were used.

In order to see how far these results were modified by the addition of salt when the beans were nearly cooked, some experiments were made in which beans were heated in boiling water for 15 minutes less than the average time required for cooking (*i.e.* 1 hr. 45 mins.), and 0·25 % of salt was then added and the heating continued until the beans were cooked. The results obtained confirmed those given in Table VI, since there was no appreciable difference in the times of cooking, whereas the loss in solids was on the average rather greater, being in fact practically the same as that observed in the case of untreated tap water.

The use of sodium bicarbonate and salt.

The results given in Sections II and III above show that the addition of sodium bicarbonate to the cooking water or preferably to the soaking water tends to decrease the time of cooking, but that the beans so cooked are somewhat insipid in flavour. The addition of salt, on the other hand, does not increase the time of cooking, improves the flavour and tends to reduce the loss of solid matter in cooking.

It therefore seemed probable that a judicious combination of the two methods would yield more satisfactory results than could be obtained by either method alone.

For this reason most of the experiments described in Section II (*A* and *B*) were repeated, the only variation made being the addition of 0·25 % of salt to the cooking water.

These experiments gave the anticipated results, and it is therefore hardly necessary to deal with them in detail.

The results as compared with those in Tables III–VI showed that the time of cooking was unaltered, the loss in solid matter on the average was slightly less and the flavour was improved.

IV. COOKING OF DRIED LEGUMES BY STEAMING.

Cooking by the action of steam instead of by boiling water is a method frequently adopted on the large scale. This method has much to recommend it from the practical point of view, but its greatest advantage lies in the fact that the loss in solid matter during cooking must necessarily be less than when the food is exposed to the action of boiling water.

The possibility of cooking dried legumes by steaming was therefore investigated.

In the first experiments made, dry beans, and also beans which had previously been soaked in water, were placed in a steamer, closed with a tightly fitting lid. This method proved to be unsatisfactory both with dry and soaked beans, as even after steaming for about four hours the beans did not swell properly and presented a dried and unappetising appearance.

It appeared, therefore, that satisfactory results could probably only be obtained when the beans were covered with water during the process of cooking. The method was therefore modified, and a series of experiments was made in which the beans were covered with boiling water and then exposed to the action of steam. The method adopted was as follows:

Method of steaming beans. 100 g. of beans were placed in a basin covered with 250 cc. of boiling water, and the basin then placed quickly in a steamer, and closed with a tightly fitting lid. The cooked beans were separated from the water by straining and weighed.

TABLE VII.

Beans cooked by steaming.

Beans (100 *g.*) *covered with boiling tap water and cooked by steaming.*

(Average results.)

Treatment of beans	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in soaking	Total percentage loss
I. Beans cooked without previous soaking	2 h. 10 m.	215	8·9	—	8·9
II. Beans cooked, without previous soaking, in water containing 0·25 % salt	2 10	217	8·4	—	8·4
III. Beans cooked, without previous soaking, in water containing 0·1 % sodium bicarbonate and 0·25 % salt	1 40	220	7·8	—	7·8
IV. Beans soaked overnight in tap water	2 5	214	8·6	1·8	10·4
V. Beans soaked overnight in water containing 1 % sodium bicarbonate and cooked in water containing 0·25 % salt	1 10	215	6·6	1·5	8·1

The water in the basin was evaporated to dryness and weighed, any necessary corrections being made as in the previous experiments.

The water below the steamer was evaporated to dryness and when allowance had been made for the solids present in the tap water, this residue was found to contain about 0·1 % of solid matter from the beans.

The results obtained in these experiments were such that it appeared worth while to pursue the matter further. In order to obtain a comparison with the results given in Tables III–VI, the experiments were repeated, using bicarbonate and also salt in the proportions previously found to be the most satisfactory. A summary of the results obtained is given in Table VII.

Discussion of results of Table VII.

These results show that although the beans were in contact with water during cooking, the loss in solid matter is less than when the beans are cooked in boiling water.

This is probably due to the fact that the water is not actually in a state of ebullition, and therefore the beans tend to break up less during the cooking process. In this connection it should be noted that the loss in solid matter observed in the individual experiments showed much less variation than in the case of boiling water (Table I). The actual values obtained in experiments (II) and (V) were as follows:

Percentage loss of solids in cooking.

	(II)	(V)
	8·5	6·6
	8·0	6·7
	8·6	6·4
Average	8·4	6·6

The time required for cooking is only very slightly greater than when boiling water is used.

The appearance and taste of the beans were considered to be superior to those cooked in boiling water, whilst as regards the action of sodium bicarbonate and of salt the results confirm the observations already made in the case of boiling water.

V. NOTE ON HARICOT BEANS AND DRIED PEAS.

Haricot beans. In order to compare the behaviour of other varieties of beans with that of butter beans, some of the experiments described in the foregoing sections were repeated using ordinary white haricot beans and also some brown beans which appeared to be fairly similar to the haricot beans but darker in colour.

The results were very similar to those obtained with butter beans. The time required for cooking was, however, slightly greater, being about 2 hrs. 15 mins.–2 hrs. 30 mins. when boiling water was used. The loss in solid matter was slightly less, namely 6–8 % of the total solids present in the beans.

Both these variations may be due to the fact that the haricot beans (white and brown) appear to have rather a thicker skin than butter beans.

Dried peas. Before discussing the behaviour of dried peas it is necessary to point out that the varieties of dried peas sold differ considerably in character. The peas sold in packets are usually smaller, have a better colour, and cook more rapidly than those sold by the pound. It is therefore difficult to make any general statement as regards the time required for cooking, and the loss in solid matter during cooking, though the latter shows an average from 6–8 % of the total solids in the peas. The experiments made show that soaking previous to cooking has a more marked effect in the case of peas than in the case of beans.

If the peas are cooked in tap water without previous soaking, the time required is, in most cases, greater than that required in the case of butter beans, the average time required with boiling water being about 2 hrs. 30 mins. Experiments carried out using the same sample of peas throughout, show, however, that the time of cooking can be reduced by about half-an-hour if the peas are soaked overnight in tap water previous to cooking.

The effect is still more marked if the peas are soaked in water containing sodium bicarbonate, since peas which required cooking for 2 hrs. 30 mins., if not previously soaked, cooked in 30 mins. after having been soaked overnight in tap water containing 1 % of sodium bicarbonate.

It is interesting to note in this connection that the effect of adding sodium bicarbonate to the soaking water appears to be much more generally recognised in the case of peas than in the case of beans, peas sold in packets sometimes containing a soaking powder or tablet. Those examined were found to consist chiefly of sodium bicarbonate, though the quantity supplied was usually rather in excess of that required. The use of sodium bicarbonate also tends to improve the colour of the peas, and it seems probable, from some experiments made with green vegetables which need not be dealt with here, that the colour is less affected by the cooking process if the peas are cooked in an open vessel, *i.e.* in a saucepan without the lid[1].

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[1] This, however, increases the time required for cooking.

As regards the action of salt and the method of cooking described in Section IV the results obtained were similar to those observed in the case of the butter beans.

VI. Suggested methods of cooking dried legumes.

In carrying out this investigation it has been assumed that the dried legumes are to be served in the whole condition. Before considering how the results obtained in the foregoing experiments can be applied to the cooking of dried legumes on a large scale, some mention should be made of other possible methods of cooking.

It is clear that, if so desired, the cooking may be prolonged until complete disintegration takes place, and the beans, together with the cooking water, may then be served as a mash. In this case there can be practically no loss in solid matter during cooking. This method, however, is not satisfactory unless the skins are removed by some process, such as passing through a sieve. The removal of the skins entails a loss in solid matter of about 6·5 %, but to what extent this loss represents a loss of food material is a matter which lies outside the scope of this enquiry.

Another method which appears to be specially favoured in the United States [Wait, 1907] is to parboil the beans in water containing sodium bicarbonate, then to remove the skins by rubbing and finally to bake the beans in an oven with the addition of fat.

This method, apart from the question of the doubtful advisability of removing the skins appears to involve too many processes to be satisfactory on a large scale, and has therefore not been dealt with in detail.

From a consideration of the results obtained in Sections I–V the following methods are suggested for cooking dried legumes on a large scale.

Method I. Soak the beans overnight or for a period of not less than four hours in water containing 1 % of sodium bicarbonate (1½–2 oz. sodium bicarbonate per gallon of water)[1] using about half a gallon of water to 2 lb. of beans. Strain off the soaking water and cook by one of the following methods:

(*A*) Place the beans in a dish or basin and cover with boiling water (about ½ gallon to 2 lb. beans) containing 0·25 % of salt (¼ to ½ oz. salt per gallon)[2] and cook in a steamer.

The time required for cooking by this process is about 1¼ hours.

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[1] The exact figure is 1·6 oz. per gallon, but the above directions are probably sufficiently accurate for working on a large scale in the kitchen. Quantities much in excess of this amount should not be added (see Section II *B*).

[2] The exact figure is 0·4 oz. salt per gallon; quantities much in excess of this amount should not be added (see Section IV).

(*B*) Place the beans in a saucepan, add tap water (1 gallon to 2 lb. of beans) containing 0·25 % of salt, and cover with a lid and cook by bringing the water to the boil.

The time required for cooking by this process is about 1 hour.

Where possible, method (*A*) should be employed since it is more economical as regards loss of solid matter in cooking besides giving more satisfactory results in other respects. Under some circumstances soaking previous to cooking may prove to be inconvenient; in such cases the beans may be cooked without previous soaking, but a longer time is then required for cooking.

Attention must, however, again be drawn to the fact that unless sodium bicarbonate is added to the soaking water there is little to be gained (except as regards colour—see Section I) by soaking the beans previous to cooking. If it is inconvenient to soak the beans previous to cooking, Method II described below may be used, but it is not satisfactory in the case of peas (see Section IV), and it should be understood that even in the case of beans Method I (*A* or *B*) is distinctly preferable.

Method II. (*A*) Place the beans in a basin or dish, cover with boiling tap water ($\frac{1}{2}$ gallon to 2 lb. beans) containing 0·1 % of sodium bicarbonate ($\frac{1}{4}$–$\frac{1}{2}$ oz. per 2 gallons of water)[1] and 0·25 % of salt and cook in steamer.

The time required for cooking by this process is about 2 hours.

(*B*) Place in a saucepan, add tap water (1 gallon to 2 lb. beans) containing salt and sodium bicarbonate in the proportions given in (*A*), cover with a lid and cook by bringing the water to the boil.

The time required for cooking by this process is about 1$\frac{3}{4}$ hours.

For the reasons stated above (*A*) should be used where possible in preference to (*B*).

The directions given above are based on experiments which have necessarily been made on a small scale, and it is possible that in carrying out the processes on a large scale it will be found necessary to introduce certain modifications.

The quantity of sodium bicarbonate used in Method II will, as already pointed out, need adjusting according to the type of water used for cooking.

On a large scale the proportion of water to beans and the time of cooking may also work out rather differently, and the above directions should be looked upon as a general guide, which by means of a few experiments made in the kitchen can be modified in accordance with the scale required and the equipment available.

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[1] The exact quantity is 0·16 oz. per gallon; quantities much in excess of this amount should not be added (see Section III *A*).

In conclusion it is desired to emphasise the fact that in carrying out this investigation primary importance has been attached to the practical aspects of the question, and it is realised that there are several factors which need to be dealt with more fully. In particular information is needed as to the composition of the residues obtained from the different cooking waters, and the nature of the action of sodium bicarbonate and salt on the dried legumes, and it is hoped to make such work the subject of further enquiry.

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XXI. SOME OBSERVATIONS ON YEAST GROWTH.

By ARTHUR SLATOR.

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It has been shown for many growths of micro-organisms that the logarithmic law of increase holds good so long as growth takes place unrestrictedly in the nutrient medium. If N is the number per cc. at time t then $\frac{dN}{dt}\Big/N$ is constant during this period of growth. This value $\frac{dN}{dt}\Big/N$, which we may call Z, is the rate of growth per cell or the constant of growth and we have the equation $Z = a$, a constant[1].

The generation-time (G.T.) $= \frac{\log 2}{Z}$.

When an old culture of bacteria or yeast is seeded into a fresh nutrient medium the initial development takes place slowly. During this lag-phase in growth Z is initially small, it increases with the time and finally reaches the value of unrestricted growth.

It has been shown by Ledingham and Penfold [1914] that the growth of *B. coli* during this period of lag follows a definite law. On calculating Z from their equation[2] we find

$$Z = at^b,$$

where a and b are constants in each experiment but vary in the different experiments with different seedings. In eight experiments b has the values 0·88, 0·77, 0·56, 0·56, 0·97, 0·74, 1·01, 1·7. It has been further pointed out

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[1] This logarithmic constant occurs in many branches of science and there are many ways of expressing it. The "constant" of a unimolecular chemical reaction, the period of "half-change" of a radioactive substance, the "birth rate" of a population, the "generation-time" in the growth of micro-organisms, "compound interest" on a sum of money, are some of the many examples which can be quoted. For a general method of recording such "velocity-constants" the inverse of the "generation-time" or the inverse of the period of "half-change" has much to recommend it and is in some respects simpler than that suggested by J. W. McBain [1917]. In this paper the usual method of giving velocity-constants is employed, but generation-times are also given.

[2] Ledingham and Penfold's equation reads $X^n = k \log Y$ where X is the time, and Y the number of bacilli (seeding $= 1$), k and n being constants. If the equation is put in the form $X^nK^n = \log Y^n$, K the new constant keeps the same value for the whole series of experiments.

Differentiating the equation we get

$$\frac{dY}{dX}\Big/Y = \frac{K^nX^{n-1}}{0{\cdot}434} = \frac{K}{0{\cdot}434}(KX)^{n-1}.$$

Substituting Z for $\frac{dY}{dX}\Big/Y$, a for $\frac{K}{0{\cdot}434}$, t for KX, and b for $n-1$, we have $Z = at^b$.

[Slator, 1917] that this equation can be slightly simplified. If a suitable unit of time be chosen the constant a in the equation $Z = at^b$ remains constant for the whole series of experiments. This equation $Z = at^b$ is not usual in chemical dynamics and it is of some interest to know whether it has any biological significance. Some interesting facts relating to growth of plants and animals are discussed by D'Arcy W. Thompson [1917, Chapter III]. The regenerative growth of tadpoles' tails seems to follow a law similar to that given above. Examples of rhythmic growth are also given.

It was in the hope of finding another example of this law that the lag phase in the growth of yeast was investigated. It was found however that this period of growth is short and does not admit of measurements such as Ledingham and Penfold have carried out. The results are described below. An account of some further experiments on yeast growth is also included.

Method of measuring the rate of growth.

Direct observation of the growth of yeast cells under the microscope was found to be most suitable for the purpose of measuring the lag-phase of growth. A hollow slide was constructed through which water at any desired temperature could be circulated. By means of such a "warm slide" the medium in which the yeast cells were growing was kept at a desired constant temperature and a number of yeast cells were continually under observation under the microscope. Other methods of keeping the slide at a constant temperature would no doubt be effective. It is evidently of some convenience to deal with a rapidly growing yeast and most of the experiments were carried out with a baker's yeast growing at 30° in malt-wort of sp. gr. 1·040.

Baker's yeast growing unrestrictedly at 30°.

Such growth was observed to take place in the following manner. A bud appears and grows steadily for about $\frac{3}{4}$ hr when it has developed to about the same size as the mother cell. For another $\frac{1}{2}$ hr no appreciable change is to be noticed. Both cells then bud usually almost simultaneously and the process of growth is then repeated. By counting the cells at various intervals of time fair constants of growth can be obtained. In the following table t = time in hours, $N + n$ = the number of cells at time t, the initial number being N. K is calculated according to the equation

$$0{\cdot}434\,K = \frac{1}{t}\log\frac{N+n}{N}.$$

Experiments 1 and 2 are single colony growths, in Exps. 3 and 4 groups of four colonies were counted.

TABLE I.

1	t	0	$1\frac{1}{2}$	$2\frac{1}{2}$	4	
	$N+n$	$4\frac{1}{4}$	10	16	35	
	$0{\cdot}434K$	—	0·248	0·230	0·229	Average = 0·236
2		Repeat exp. gave				,, = 0·244
3	t	0	1	$1\frac{3}{4}$	3	
	$N+n$	$9\frac{3}{4}$	17	$28\frac{1}{4}$	57	
	$0{\cdot}434K$	—	0·241	0·264	0·256	,, = 0·254
4		Repeat exp. gave				.. = 0·248

Average of the four = 0·246, G.T. = 1·22 hrs = 73 mins.

Another method of measuring this generation-time gave good results. It is carried out in the following manner. A yeast cell is observed to bud and the time t_1 noted. The bud grows and eventually two new buds appear, one on each cell usually within a few minutes of each other (Fig. 1). The mean time t_2 is observed. The difference $t_2 - t_1$ is the time of a complete generation. The method is suitable for estimating the G.T. of individual cells. Twelve cells watched in this way under the microscope gave values 78, 66, 76, 70, 79, 65, 81, 72, 72, 70, 72, 73 the average being 73 mins. The G.T.s of individual cells vary therefore up to 8 mins on each side of the average.

TABLE II.

Cell number	Time of appearance hrs mins	Average hrs mins	Time when fully grown hrs mins
2	0 0	0 0	0 45
3	1 18		
4	1 16	1 17	2 0
5	2 33		
6	2 33		
7	2 33		
8	2 33	2 33	3 15
9	3 49		
10	3 50		
11	3 52		
12	3 38		
13	3 45		
14	3 48		
15	3 52		
16	3 38	3 47	

Generation-times = 77, 76, 74, average 76 mins.

The following case of a single cell growing to sixteen cells is of interest. The time at which each cell appeared as a bud was noted and each successive average G.T. obtained from the time differences. In the table the number of each cell corresponds to that given in the diagram (Fig. 2).

The lag-phase.

If an old culture of yeast is seeded into wort an hour to two hours elapses before visible growth takes place. It is then found that growth proceeds at the maximum rate.

A five days old culture of yeast was seeded into fresh wort and the G.T. of thirteen cells estimated as soon as buds appeared. They gave values 81, 89, 80, 78, 77, 72, 85, 74, 88, 78, 76, 81, 70 mins; average 81 mins, a value only slightly greater than the minimum G.T. When older cultures were used similar results were obtained but more dead cells were present.

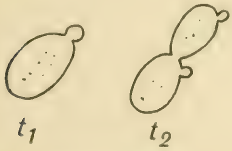

Fig. 1. Generation-time $= t_2 - t_1$.

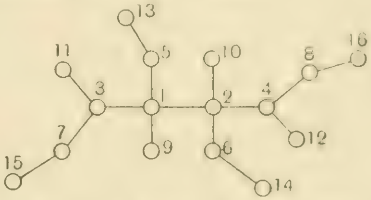

Fig. 2. Diagram of yeast growth.

Individual cells show considerable variation in the time they remain quiescent before budding. Thirty-seven cells kept under observation gave the following results:

During the first 60 mins.		0 budded	from 91 to	95 mins.	2 budded
from 60 to	65	6	96	100	4
66	70	7	101	105	0
71	75	4	106	110	1
76	80	8	111	115	1
81	85	1	116	120	1
86	90	1	121	125	1

The lag-phase in the growth of a yeast cell consists therefore of a quiescent stage of one or two hours varying with the individual cells and then growth at the normal unrestricted rate takes place.

Yeast growth proceeds in alternate periods of growth and rest and the lag is apparently little more than a prolonged period of rest. Many attempts have been made to deduce the equation $Z = at^b$ from such data but without success.

If it were possible to follow the growth of *B. coli* under the microscope and compare it with the results obtained by plating, an explanation of Ledingham and Penfold's equation might be forthcoming. It is worth noting that the death of some of the cells may take place during such measurements. Thus Penfold [1914, Table X, p. 230] records an initial decrease in the number of bacilli in some cases; that is a certain number of the original seeding die in the nutrient medium but grow if gelatin is present. The presence of a poison which diffuses slowly might account for the favouring influence of gelatin.

The growth of spores.

It was thought possible that the development of yeast spores might give a longer period of lag than is the case with quiescent yeast cells. Experiments were therefore made with a wild yeast which spores abundantly. Measurements of the G.T. of the yeast growing in malt-wort at 30° gave values 91, 82, 84, 81, 92. 95 mins, average 88 mins. When spores are kept under observation on the warm slide the general characteristics of spore growth are observed: the long period of rest (often 24 hrs) before visible development, the bursting of the cell walls and the budding of the spores. This wild yeast developed at first small round cells which grew in a mass and could not be counted. After about the second generation the normal larger cells developed, the rate of growth of which was easily estimated. Three cells gave values 94, 85, 88 mins. that is the maximum rate of growth is already attained. There is therefore little or no lag when once the spores have started developing.

Influence of carbon dioxide.

To obtain the maximum rate of yeast growth (minimum G.T.) it is essential that there should be no appreciable accumulation of carbon dioxide in the nutrient medium. This is the case if the number of yeast cells does not exceed a few hundred thousand per cc. That carbon dioxide has an appreciable retarding influence is easily demonstrated. If the experiments are carried out with wort saturated with carbon dioxide as the nutrient medium, a number of the cells are observed to die and those which survive grow at about half the maximum rate. Yeast which has been growing some time in the presence of carbon dioxide no longer exhibits the dying off effect and grows rather faster, but in no case was the maximum rate of growth observed. The effect of carbon dioxide on yeast growth is well known and seems to be general. Air which has a large influence in the later stages of yeast growth has no influence when dealing with these small concentrations of yeast, for rates of growth measured under these conditions in wort saturated with air and in wort from which the air had been evacuated gave the same result.

Yeast growth at high temperatures.

If rates of growth at different temperatures are plotted against the temperature a characteristic curve is obtained. Below a certain low temperature and above a certain high one no growth takes place. Between the two the rate of growth varies greatly. As the temperature rises the rate increases and finally attains a maximum. A further increase in the temperature causes a rapid decrease in the rate which finally becomes zero. In an investigation of the influence of temperature on the rate of growth of *B. delbrücki* it was observed that the rate was about halved when the temperature was raised from 50° to 52·5° [Slator, 1916]. The rate however remained constant at the higher temperature. It is of interest to know whether the lower rate is due to cell destruction or to slower rates of growth of the individual cells. By observing growth on the "warm slide" this point can be settled as far as yeast cells are concerned. The baker's yeast used in these experiments gave an average G.T. of 63 mins at 39° to 40°. At 41° retardation in growth takes place and some of the cells also die. Observations of a group of four cells growing at 41° gave the following result, one cell died before budding and the other three gave G.T. 67, 77, 85 mins. Other groups of cells showed similar results—some dying and some growing. At these high temperatures therefore some of the cells grow at about the maximum rate, some at a considerably lower rate and some die.

The rate of spore production in yeasts is influenced by temperature in a similar manner to growth. These rates are usually measured in the following way. An actively growing culture is spread over a moist gypsum block and kept at a constant temperature. An examination is made periodically and the time of the first appearance of spores is noted. The times observed are therefore the minimum for spore production at the given temperature. If one constructs curves plotting temperature against the inverse of these times the similarity to temperature-rate of growth curves is very noticeable. Here again we have the upper and lower limiting temperatures, a maximum rate, and a part of the curve where the temperature coefficient is less than one. We are dealing here only with the few cells which form spores in the shortest possible time and the slower rates at high temperature cannot be explained by cell destruction. Probably both with spore production and with growth at temperatures above that of maximum rate partial destruction of some substance affecting the rate takes place but is not sufficient to stop development entirely.

In the case of enzyme action where a similar maximum occurs temperature coefficients less than one are usually attributed entirely to destruction of the enzyme.

Other uses of the "warm slide."

This method of investigating yeast growth can be conveniently used in all cases when rapid growth takes place. It is also of use in determining the initial effect of poisons on growth, thus a dying off effect or a lag-phase is soon detected by this means whilst they may be missed entirely if rates of growth are estimated by ordinary counting methods.

Use of the "warm slide" was made to throw some light on the effect of yeast growth on diastatic action. The experiments showed no new results but they suggested a point which is worth mentioning. Diastase (malt extract) has a slow solvent action on malt starch at ordinary temperatures and this reaction is accelerated if yeast is present. G. H. Morris [1901] showed that the products of reaction of diastase on starch have no influence on the rate of the reaction and it is unlikely therefore that removal of the reaction products by fermentation would account for the acceleration. The action of diastase on starch is accelerated by carbon dioxide [for references see Euler. 1912, p. 158] and no doubt the carbon dioxide evolved by the yeast influences the reaction. Whether this effect accounts for the acceleration entirely requires other experiments to prove but it certainly has to be taken into consideration.

The influence of oxygen on yeast growth.

The action of air (oxygen) on yeast growth has attracted attention from the time of Pasteur onwards and rightly so, for oxygen plays an important part in yeast growth. One of the most striking ways of showing that air is necessary to yeast growth is to attempt to grow "giant colonies" of yeast on wort gelatin in an evacuated vessel. Growth is very poor and liquefaction of the gelatin soon takes place under these anaerobic conditions.

Within the last few years H. T. Brown [1909, 1914] has more thoroughly investigated this influence of oxygen. In agreement with A. J. Brown [1905] he shows that when yeast develops in malt-wort, growth is retarded and finally ceases at an earlier stage than one would expect taking into consideration the food requirements of the yeast and the retarding influence of alcohol and carbon dioxide on growth. He concludes that lack of oxygen is the main factor which causes this retardation. The experiments described here are in accordance with this conclusion. He further finds that very considerable yeast growth takes place under strictly anaerobic conditions. This admits of explanation on the assumption that there is a certain amount of chemically combined oxygen in the wort which is available for yeast growth. Subsequently [1914] H. T. Brown discards this explanation and considers that the yeast used for seeding carries oxygen with it in the form of an "oxygen charge" and that growth is determined by this "charge."

The theory gives a ready explanation of the approximately rectilinear curves of growth to which he draws attention. The alternative explanations depend on whether retardation in growth is due to change in the medium (lack of combined oxygen) or to change in condition of the yeast (continual splitting of the "oxygen charge").

TABLE III.

Aerobic growth at 20°

t (hrs)	$N+n$	$0{\cdot}434K$
0	11,500	—
22·5	630,000	0·077
42	11,200,000	0·071
0	226	—
42½	175,000	0·068
47	441,000	0·070
0	4·4	—
66½	365,000	0·074
		0·072

G.T. = 4·2 hrs

Anaerobic growth at 20°

t	$N+n$	$0{\cdot}434K$
0	11,500	
42 hrs	322,000	0·034
0	226	—
4¾ days	266,000	0·027
5 ,,	1,440,000	0·032
0	4·4	—
9 ,,	7,000,000	0·029
		0·031

G.T. = 9·7 hrs

Estimations of the rate of growth with very small seedings were made to decide between these two alternatives. The experiments described below show fairly conclusively that there is in malt wort combined oxygen available for yeast growth. Rates of growth were estimated by yeast counts according to Method 1 previously described [Slator, 1913], a Burton brewery yeast being used for the purpose. It grew at a rate corresponding to a G.T. of 3 hrs at 20° in malt-wort of sp. gr. 1·049, and the same results were obtained whether the experiments were carried out in the presence of air or in evacuated tubes. All attempts to obtain lower rates by drastically excluding air were unsuccessful. Efforts were then made to find a medium in which growth is influenced by air. Half-fermented wort in which yeast growth had stopped was filtered and partly evaporated in an open dish to free it from alcohol and growth in this medium tested. It was found however that yeast grew in this medium both anaerobically and aerobically at the same rate. Wort of sp. gr. 1·060 was then taken, fermented until it contained about 2 % of alcohol and filtered out of contact with air. It was then evaporated in an evacuated distilling flask to bring it back to about its original gravity of 1·060. In this medium yeast behaved differently. Rates of growth under anaerobic conditions were much smaller than under aerobic conditions. Table III gives the results of measurements of rates of growth with different seedings: in one case the experiments were carried out with free access of air, in the other under anaerobic conditions in a number of evacuated tubes. A G.T. of 4·2 hrs was observed when air was present and a G.T. of 9·7 hrs when air was absent. The seeding in each case was made with yeast which was grown under anaerobic conditions. The presence of air was found to favour the growth of smaller cells than were produced when air was absent, but as the increase over the period of growth is great the difference in the size of the cells would hardly affect the velocity-constants.

There is no doubt that partly fermented wort differs from the original wort in that combined oxygen available for yeast growth is present in the one case and very much reduced in the other. The effect of oxygen on yeast growth deserves more attention than is given in this short note, but apparently dissolved oxygen (which is rapidly absorbed by the yeast) and oxygen chemically combined in the wort are both available for yeast growth. The necessary amounts are small; thus Brown [1914] estimates that 1·7 cc. of oxygen are used up in the growth of 10^{10} cells (about 2·5 g. of pressed yeast).

SUMMARY AND DISCUSSION OF RESULTS.

A method of measuring rates of growth of yeast by direct observation under the microscope is described. The G.T. of individual cells of a baker's yeast growing in malt-wort at 30° varies up to 10 % on each side of the average value of 73 mins.

Measurements of the lag-phase in the growth of yeast cells show that old yeast cells remain quiescent for some time when introduced into a fresh medium, the time of quiescence varying with different cells. They then start growing at the normal unrestricted rate. Yeast growth proceeds in alternate periods of rest and growth, and lag is apparently little more than a prolonged period of rest.

Little or no lag is observed in yeast cells grown from spores, when once development has started.

The retarding influence of carbon dioxide is readily observed by this method of measuring growth.

At a temperature above that of maximum rate of growth some cells grow at about the maximum rate, some at lower rates and some die.

The influence of oxygen on yeast growth has been tested by measuring generation-times under anaerobic and aerobic conditions. The results confirm previous conclusions that air is necessary for yeast growth, and that oxygen chemically combined in the wort as well as dissolved oxygen which is rapidly absorbed by the yeast is available for yeast growth. A method of preparing a medium in which yeast growth is sensitive to air is described.

If yeast is seeded into malt-wort the following stages of growth may be observed: the lag-phase or period of quiescence, the logarithmic phase or period of unrestricted growth, retardation in growth due to carbon dioxide, retardation in growth due to failure of oxygen. The latter factor is the main one which finally stops growth. The accumulation of alcohol and lack of necessary nutrients seldom come into consideration up to this stage. When fermentation is over air may have access and cause a regrowth of the yeast, the alcohol and possibly lack of nutrients such as assimilable sugar are then the main retarding factors.

It depends on conditions of seeding, aeration, temperature, etc., how far these different stages of growth become prominent, overlap or disappear. Thus if the seeding consists of actively growing yeast the lag-phase disappears, if the seeding amounts to a few million per cc. retardation starts at once and there is no logarithmic period, if the seeding is very great lag in growth and rapid accumulation of retarding influences may prevent growth entirely. The problem of the final yeast crop is largely a matter of rates of growth with failing supplies especially of oxygen.

Thanks to the work of A. J. Brown, A. L. Stern [1899, 1901], H. T. Brown and others it should not be difficult to determine rates of growth with definite small concentrations of known essential nutrients and possibly put the problem of yeast crops on a sound mathematical basis.

In this and in previous papers [1913, 1916] several methods of measuring the logarithmic constant of growth and the generation-time of micro-organisms have been described, and attempts have been made to show the importance of these measurements in an investigation of the growth of micro-organisms and the chemical action brought about by them. The figures obtained are not merely interesting mathematical curiosities. They represent accurate measurements of growth under conditions where *the medium remains practically unaltered*, and they are of considerable value in solving problems connected with the growth of micro-organisms. It may be said that yeast (for instance) for practical purposes never grows under such conditions and that measurements under "normal" conditions are of more value. The argument is only partly correct for one can investigate phases of retarded growth (such as when yeast growth is retarded by lack of oxygen) by this method, and in general it is better to study each part of the puzzle under the simplest possible conditions and then attempt to fit the pieces together into a harmonious whole. The method is in fact that of investigating growths of micro-organisms and the chemical actions brought about by them in the same manner as one would investigate the kinetics of a purely chemical reaction. One has every confidence that the method will justify itself in throwing fresh light on these problems. Much of the work carried out in connection with the fermentation industries especially relating to growths of yeast, acetic acid bacteria, lactic acid bacteria, etc., is sufficiently far advanced to be investigated from this point of view and such measurements would be well worth making. The field of investigation is by no means limited to these organisms but extends to all branches of science in which micro-organisms play a part.

In conclusion I desire to express my thanks to Dr A. L. Stern, with whom I have had the advantage of discussing the subject matter of the present paper.

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XXII. THE ANTISCORBUTIC FACTOR IN LEMON JUICE.

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In a previous communication [1918] we have described an attempt to isolate the antiscorbutic principle by means of adsorption. The attempt proved unsuccessful but we were able to make a series of observations in connection with the behaviour of the antiscorbutic factor in orange juice towards some adsorbents under certain conditions. We have since attempted to study the chemical nature of the factor in question adopting another method of investigation, namely that of fractionating an antiscorbutically active juice. We have chosen lemon juice for that purpose. As is well known the main bulk of the solids of lemon juice consists of citric acid. In the event of the antiscorbutic being associated with the citric acid, good yields of the acid could be obtained from the juice for further investigation. If on the other hand by removing the citric acid the solid residue remained active, the antiscorbutic would then be associated with a comparatively much smaller mass of extraneous matter offering a better opportunity of studying both its chemical and physiological properties. The latter has proved to be the case and we now describe in this communication a few experiments from an investigation in progress on the above lines.

Experimental.

The following procedure was adopted in order to remove the free citric acid from lemon juice. To freshly squeezed out lemon juice an excess of precipitated calcium carbonate was added with constant stirring. The resulting suspension was then treated with two volumes of absolute alcohol and filtered through a fluted filter. The residue remaining on the filter was then pressed out in a hand press and the liquid thus obtained filtered. Both filtrates, which should be clear, were combined and evaporated in vacuo at 35° C. until all the alcohol had been removed and were then made up with distilled water to the original volume of the lemon juice used. The solution thus obtained is very slightly acid to litmus, yellow in colour, sweetish to taste and retains the characteristic lemon flavour as well as the bitter taste of the lemon. On evaporating the solution to dryness and heating the residue at 110° for an hour the equivalent of 1 cc. of the original lemon juice yields a residue weighing about 15 mgms. The residue after heating becomes caramelised and syrupy and tastes very sweet. It reduces Fehling's solution freely. On incinerating the residue about 3 mgms. of inorganic constituent per cc. of original lemon juice is obtained. The inorganic residue resulting from 10 cc. lemon juice treated as above when dissolved in a known quantity of decinormal H_2SO_4 and titrated back required 2 cc. of $N/10$ H_2SO_4 to neutralise the carbonates produced by the incineration of the small quantities of the salts of organic acids present and this when calculated on the basis of sodium citrate corresponds to 1·7 mgms. per cc. When the decitrated juice was treated with $CaCl_2$ and two volumes of absolute alcohol a scanty precipitate of a few mgms. was obtained from 10 cc. of the solution and this consisted of the calcium salt of some organic acid or acids, as it charred and smelled of burning sugar when heated. A sample of lemon juice evaporated to dryness and heated at 110° for one hour gave a total residue of 105 mgms. per cc., 4 mgms. of which was inorganic.

Experiments were instituted to test the therapeutic and other properties of the treated lemon juice.

Expt. 1. The purpose of this experiment was to test the antiscorbutic potency of the treated lemon juice. Quantities equivalent to 2 cc., 3 cc., 5 cc. and 7 cc. were tried respectively on four guinea pigs. The animals received oats and bran *ad lib.* and 50 cc. of autoclaved full milk per diem. The respective doses of the treated lemon juice were administered daily by hand. The animals were allowed to go on for about eighty days when they were all chloroformed. It will be seen from fig. 1 which represents the weight curves of the animals, that on 2 cc. guinea pig No. 71 just managed to maintain its weight. Although it was unable to grow on that dose it failed nevertheless to succumb to scurvy. The other animals in the experiment actually succeeded in putting on weight. At the post mortem examination guinea pig No. 72, which received a dose of 3 cc. showed slight haemorrhages in the femoral muscles and also slightly enlarged costochondral junctions. The other animals (Nos. 54, 55 and 71) showed no signs of scurvy at all. This experiment shows without any doubt that after removing the free citric acid the residual fraction of lemon juice retains some antiscorbutic activity.

Expt. 2. Our next aim was to ascertain approximately what proportion of the antiscorbutic content of lemon juice remains in the residue after the removal of the acids. It has previously been pointed out by us in another communication [1918] that with our present technique it is impossible to carry out quantitative determinations of the antiscorbutic content of a solution with any great precision. It is nevertheless possible to ascertain roughly the relative antiscorbutic potency of two solutions by administering equal quantities of the solutions to guinea pigs of approximately the same weight kept on a scorbutic diet and watching the clinical developments. We have adopted this procedure in this experiment. Doses of 0·5 cc., 1 cc. and 1·5 cc. each of treated and untreated lemon juice respectively were given to six guinea pigs. Animals Nos. 263, 267 and 268 received 0·5 cc., 1 cc. and 1·5 cc. of untreated lemon juice respectively, while Nos. 266, 264 and 265 received 0·5 cc., 1 cc. and 1·5 cc. of treated lemon juice. Fig. 2 represents their weight curves. Guinea pigs Nos. 263 and 266 receiving 0·5 cc. of untreated and treated lemon juice respectively died of scurvy within about a month, showing that 0·5 cc. either of treated or untreated lemon juice is insufficient to prevent guinea pigs from succumbing to scurvy. No marked differential clinical features could be observed in these animals. The behaviour of Nos. 264 and 267 which received a dose of 1 cc. was also very similar. They were both chloroformed

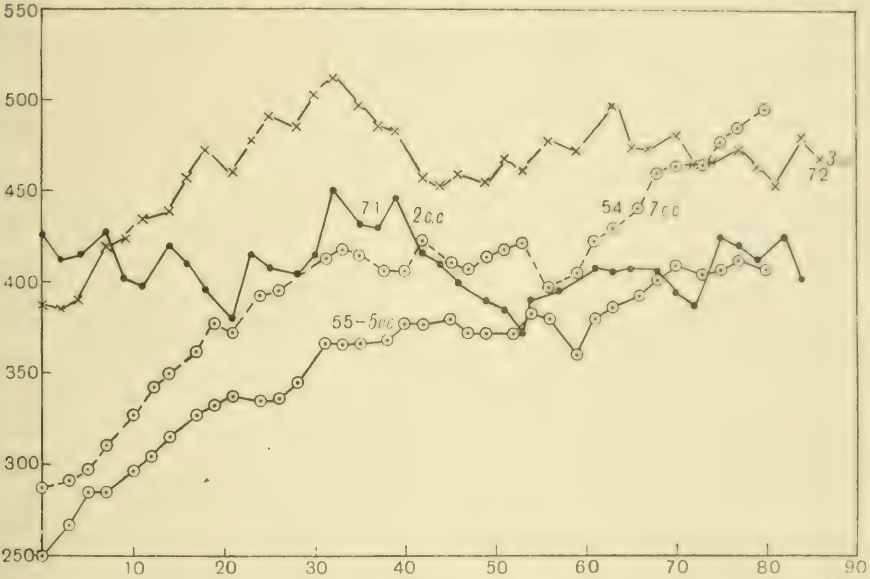

Fig. 1

after forty-two days and at the post mortem they both disclosed signs of scurvy. The weight curve of No. 267 which received the untreated lemon juice is however a little better than that of No. 264 which was dosed with the treated juice. Guinea pigs Nos. 265 and 268 received 1·5 cc. of treated and untreated lemon juice respectively. Neither of these animals developed scurvy. Unfortunately No. 268 succumbed after thirty-seven days to an intestinal complaint, which is often the case with guinea pigs receiving high doses of lemon juice. Not the slightest trace of scurvy could however be found at the post mortem examination. No. 265 was chloroformed after forty-two days and was found to be in excellent condition. Nothing abnormal was disclosed by the post mortem examination. It is seen then that the three sets of pigs behaved almost identically, showing that the relative antiscorbutic potency of the treated and untreated lemon juice is practically the same, from which one may safely conclude that little if any of the antiscorbutic is associated with the free acids in the lemon juice of the solids of which they form the main portion.

Expt. 3. We endeavoured next to ascertain whether treated lemon juice is antiscorbutically stable for short periods of time. Small portions of treated lemon juice were stored in the cold room in corked flasks for about a fortnight before being tested. Quantities of 3 cc., 5 cc. and 8 cc. were tried on three guinea pigs. As will be seen from fig. 3 all the animals failed to succumb to scurvy within seventy-four days, after which period they were chloroformed. No. 76 which received 8 cc. and No. 74 which received 3 cc. of the treated extract showed very slight signs of scurvy at the post mortem examination. The other guinea pig was quite normal. As the weight curves show, none of the animals grew satisfactorily. The stored juice then seemed to have deteriorated to some extent during the fortnight's cold storage although it retained the best part of its antiscorbutic potency.

Expt. 4. It was of interest to see whether on evaporating the treated lemon juice the residue would retain its antiscorbutic power. We therefore evaporated a litre of the treated lemon juice at a temperature of 30°–40° C. in vacuo until the residue assumed a thick syrupy consistency. The residue was then transferred to an evacuated desiccator where it was kept during the experiment. Three pigs, Nos. 68, 69 and 70, received 100 mgms., 150 mgms. and 200 mgms. per diem of the residue respectively in addition to the scorbutic diet. The doses were dissolved in suitable quantities of distilled water just before administration. All the pigs died of scurvy within 42–50 days. Fig. 4 represents the weight curves of the above animals. It is seen that the doses given have

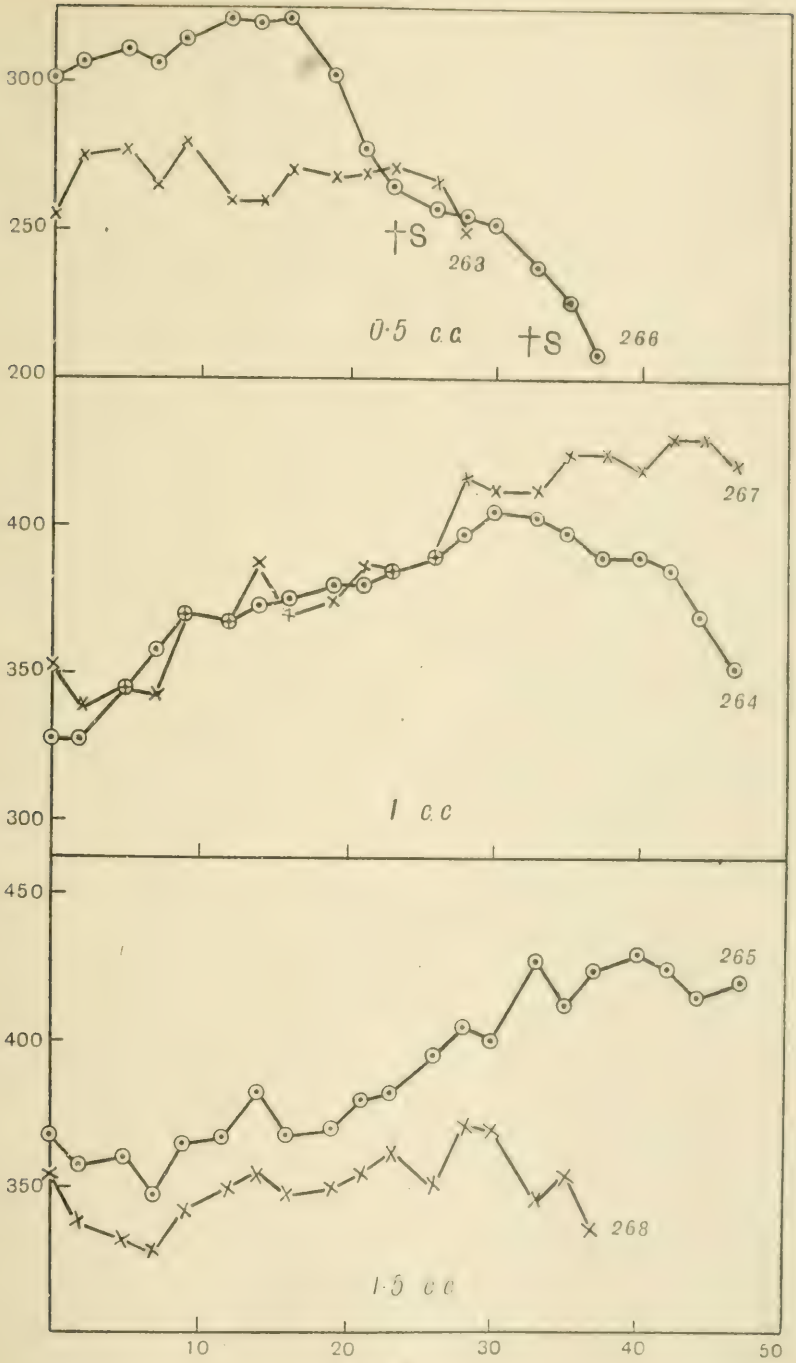
300
250
200
†S
263
0·5 c.c.
†S
266
400
350
300
267
264
1 c.c
450
400
350
265
268
1·5 c.c
10
20
30
40
50

Fig. 2

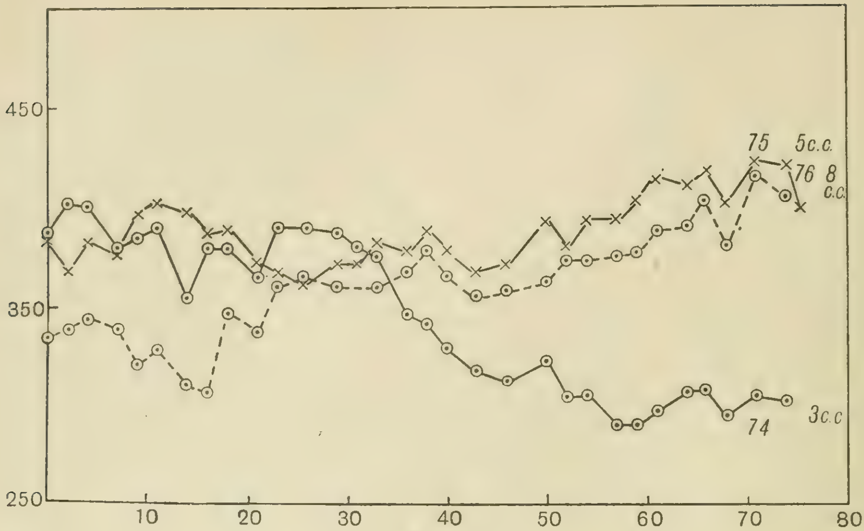
450
350
250
10
20
30
40
50
60
70
80
75
5c.c.
76
8 c.c.
74
3c.c

Fig. 3

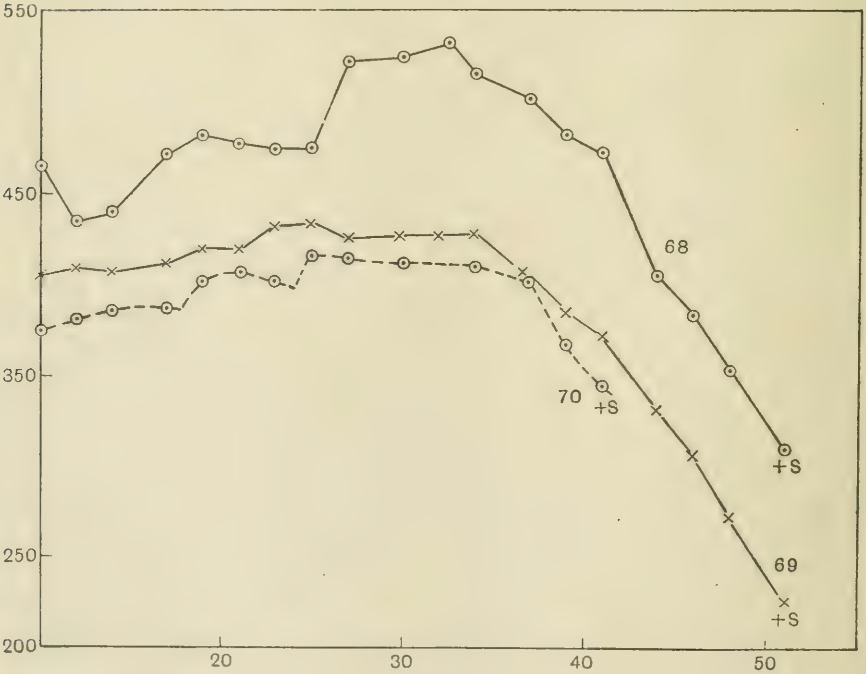
550
450
350
250
200
20
30
40
50
68
70
+S
+S
69
+S

Fig. 4

delayed slightly but not prevented the onset of scurvy. As the doses given were comparatively high it is plain that by drying the treated lemon juice in the way we have just described the best part of its antiscorbutic activity is destroyed.

Expt. 5. From experimental evidence at our disposal obtained in the course of another investigation we decided to modify the process of desiccation by previously acidifying the treated lemon juice. After the precipitation of the free acids of the lemon juice with $CaCO_3$ and alcohol 1 gm. of citric acid was dissolved in the alcoholic filtrate of 1 litre of lemon juice. It was then evaporated *in vacuo* at 30°–40° as before. A residue of 14 gms. was obtained.

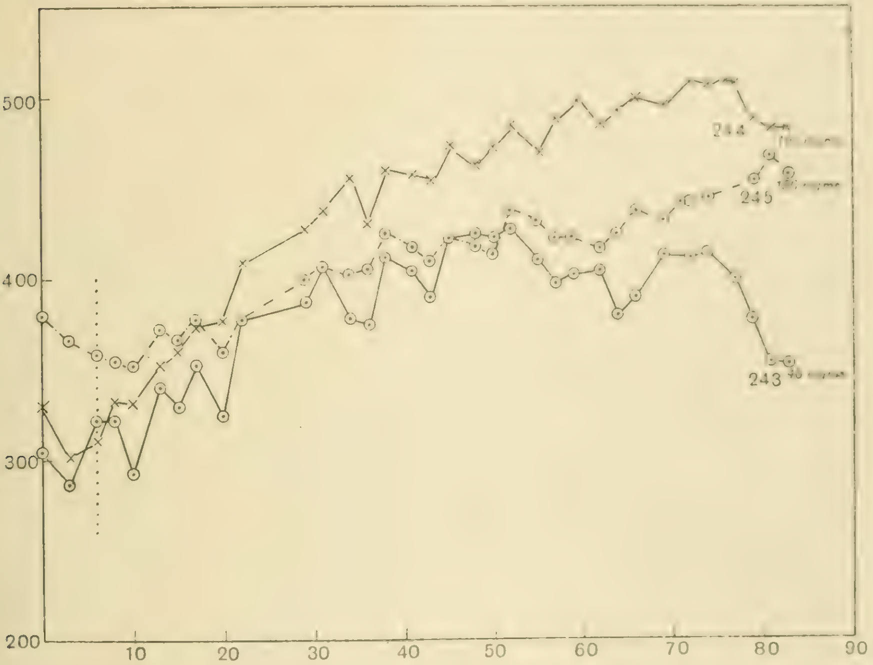

Fig. 5

Guinea pigs Nos. 243, 244 and 245 received 45 mgms., 105 mgms. and 150 mgms. respectively besides the usual scorbutic diet. As before the dry residue was dissolved before administration. As will be seen from fig. 5 which represents the weight curves of these animals they were all increasing in weight. After eighty-three days the animals were chloroformed and at the post mortem examination were found to be in excellent condition. Not the slightest signs of scurvy were to be observed. It is thus seen that by acidifying slightly the medium, treated lemon juice can be evaporated to dryness yielding a residue of high antiscorbutic potency.

Expt. 6. When pigeons suffering from an acute attack of polyneuritis are treated with subcutaneous or intramuscular injections of highly potent antineuritic extracts a cure is brought about even with greater rapidity than when the treatment is carried out *per os*. Holst and Frölich [1912] administered cabbage juice intraperitoneally to guinea pigs subsisting on a scorbutic diet as preventive doses but were unsuccessful in preventing the onset of scurvy from which their animals ultimately died. As the treated lemon juice described above contains very little extraneous matter highly potent doses can be administered. It was of interest to see if by injecting concentrated doses of the treated juice subcutaneously into guinea pigs suffering from scurvy, the animals could be cured. Three guinea pigs were employed for the purpose and the following protocols describe the experiment.

Guinea pig No. 67. When put on a scorbutic diet it weighed 261 g. After subsisting on this diet for nineteen days it received a subcutaneous injection of 15 cc. of concentrated treated lemon juice equivalent to 100 cc. of the original lemon juice. The weight of the animal kept on decreasing in the usual way after the treatment and it died two days later, weighing 271 g. The post mortem examination revealed a very acute condition of scurvy.

Guinea pig No. 58 A. When put on scorbutic diet it weighed 467 g. After twenty-one days it received a subcutaneous injection of 10 cc. of concentrated treated lemon juice equivalent to 100 cc. of the original lemon juice. The weight kept on decreasing and the animal died four days after the treatment. The post mortem examination revealed an acute condition of scurvy.

Guinea pig No. 66. When put on a scorbutic diet it weighed 845 g. After twenty-five days it received a subcutaneous injection of 10 cc. of concentrated treated lemon juice equivalent to 100 cc. of the original lemon juice. The weight of the animal kept on decreasing and it died three days later. The post mortem revealed a very mild condition of scurvy.

It is seen from the above experiments that when guinea pigs are suffering from well declared scurvy subcutaneous injection of concentrated doses of an antiscorbutic does not produce any amelioration in the condition of the animal and the progress of the disease is unaltered.

Expt. 7. This experiment was instituted with the purpose of ascertaining whether by administering high doses of antiscorbutic before putting animals on a scorbutic diet any prophylaxis could be achieved. By using concentrated doses of treated lemon juice very large quantities of very high potency could be administered within a few days which it would have been impossible to give with the corresponding original lemon juice. The following protocols describe the actual experiment.

Guinea pig No. 80 received within four days concentrated doses of treated lemon juice equivalent to 1280 cc. of original lemon juice. It was then put on a diet of oats, bran and autoclaved milk. Its weight at the commencement of the experiment was 375 g. After fourteen days it began to decrease in weight and died after it had existed twenty four days on the scorbutic diet. At the time of its death it weighed 259 g. The post mortem examination revealed acute scurvy.

Guinea pig No. 81 received within five days concentrated doses of treated lemon juice equivalent to 1050 cc. of original lemon juice. Its weight at the time it was put on a scorbutic diet of oats, bran and autoclaved milk was 359 g. It began to decrease in weight after eighteen days and died twelve days later. At the time of its death it weighed 199 g. The post mortem examination revealed acute scurvy.

Guinea pig No. 82 received within four days concentrated doses of treated lemon juice equivalent to 900 cc. of the original lemon juice. It was then put on a scorbutic diet of oats, bran and autoclaved milk. Initial weight was 358 g. After sixteen days it commenced to decrease in weight and died eight days later. Its final weight was 238 g. The post mortem examination revealed very acute scurvy.

The administration then of high doses of antiscorbutics previous to a period of deprivation of the antiscorbutic factor does not prevent or delay the onset of scurvy in guinea pigs, as the protocols show that the animals behaved in the same way as animals kept on a scorbutic diet without receiving any previous treatment.

Expt. 8. It was shown by Hart and Lessing [1913] that scurvy can be induced in monkeys. The clinical picture and course of the disease in these animals is almost identical with that of human beings. We too have managed to induce scurvy in monkeys by using a diet which was theoretically complete in every respect but lacked the antiscorbutic element and the description of these experiments will be communicated in detail elsewhere. In view of the fact that all our experiments had been carried out on guinea pigs it was also desirable to study the curative action of the treated lemon juice on a scorbutic monkey. A *Macacus rhesus* monkey, estimated to be about two years of age, suffering from acute experimental scurvy was submitted to the treatment. The details of the experiment are given in the following protocol.

Monkey No. 6 (female *Macacus rhesus*) was put on a scorbutic diet on October 18. Incipient symptoms of scurvy observed on December 29. From that time the disease became gradually worse. On January 29 the animal was in an acute scorbutic condition showing haemorrhaged eyelids, loose teeth with disintegrating enamel. The gums very spongy and haemorrhaged. The animal was almost unable to use its legs. It then received a dose of treated lemon juice by means of the stomach pump. For the following five days it received convenient doses of the treated lemon juice which amounted altogether to the equivalent of 980 cc. of the original lemon juice. On January 24, the day following the administration of the first dose, the teeth became clearer. On January 25 the condition of the gums was markedly improved and the teeth were decidedly firmer. The improvement continued and by January 28, five days after the commencement of the treatment, the gums were normal, the teeth almost firm and the animal was using its legs with alacrity. The condition of the animal soon became normal. It was now receiving a daily dose of treated lemon juice equivalent to 10 cc. of the original juice besides the original scorbutic diet. The monkey throve well on this supplemented diet. On March 16 it was chloroformed and at the post mortem examination, with the exception of a deformed wrist which was evidently the vestige of a fracture which had taken place in the acute scorbutic condition, everything was found to be normal. The histological examination of the costochondral junctions in the ribs did not disclose any perceptible abnormality.

This experiment definitely confirms the potent antiscorbutic activity of the treated lemon juice described in this communication.

SUMMARY.

1. By eliminating the free citric and other organic acids in lemon juice an antiscorbutically active residue is obtained.

2. Comparative experiments show that the best part if not the whole of the antiscorbutic content of the lemon juice is present in this fraction.

3. After storing the treated lemon juice for about a fortnight in the cold room a marked loss can be observed in its potency although it still remains fairly active.

4. By evaporating the treated lemon juice to dryness in acid medium an active dry residue is obtained.

5. Subcutaneous injections of very potent doses of treated lemon juice failed to arrest the progress of well declared scurvy in guinea-pigs.

6. The administration of very concentrated doses of treated lemon juice previous to depriving guinea pigs of the antiscorbutic factor did not prevent or delay the onset of scurvy.

7. The very high curative effect of the treated lemon juice on a scorbutic monkey has been demonstrated.

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XXIII. NOTE ON THE ETIOLOGY OF SCURVY IN GUINEA-PIGS.

By ARTHUR HARDEN and SYLVESTER SOLOMON ZILVA.

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(*Received July 5th, 1918.*)

As a result of experiments carried out on rats and guinea-pigs McCollum and Pitz [1917] came to the conclusion that scurvy in the guinea-pig is not a disease brought about by the deficiency of an accessory factor in the diet but that the scorbutic condition is caused by a diet chemically and physically unfavourable. It is particularly the physical condition of the scorbutic diet of oats which is supposed by them to have a detrimental effect on the digestive tract of the guinea-pig, causing constipation and thus facilitating the absorption of toxins produced by the bacterial flora of the intestine. These workers were unable to produce scurvy in rats by feeding them on diets of which oats formed the basal constituent, they were however able to induce scurvy after a certain period only in some of their guinea-pigs which were fed on rolled oats and fresh whole milk. Adopting that technique McCollum and Pitz tried a variety of substances which were intended either to obviate constipation in their experimental guinea-pigs or to attenuate the bacterial development in the alimentary tract of the animals. As some of the substances tested produced a beneficial result the authors considered their hypothesis to be substantiated by their observations. Such conclusions however are not in our opinion warranted by the experimental evidence given in their paper. Fresh whole milk was given to the animals *ad lib.* Milk if not subjected to any heating is antiscorbutic and it has been shown recently by Chick, Hume and Skelton [1918] that if large enough quantities of fresh milk are taken by guinea-pigs scurvy does not supervene. We agree with the suggestion of the last mentioned investigators that had the quantities of milk consumed by the animals in McCollum and Pitz's experiments been measured, the beneficial effect observed in their animals treated with the various substances could in all probability have been traced to the antiscorbutic potency of the augmented quantities of fresh milk consumed.

McCollum and Pitz, in the same communication, ascribe the antiscorbutic activity of orange juice to its content of sodium and potassium citrates which possess laxative properties and to the retardation of the development of bacteria by the action of citric acid contained in the juice. These explanations appear hardly feasible in view of the observations made by us in connection with the antiscorbutic content of lemon juice. In the preceding paper [Harden and Zilva, 1918] we have shown that by removing the free citric acid from lemon juice a residue is obtained whose inorganic content is almost insignificant, which nevertheless contains almost the entire antiscorbutic content of the original juice. It is therefore evident that the activity of the extract cannot be ascribed either to its content of citric acid or of sodium and potassium citrates as the inorganic residue of the preventive dose of 1·5 c.c. only corresponds to about 2–3 mg. of potassium or sodium citrate.

Pitz [1918] in a recent communication describes experiments which in his opinion offer further evidence in support of the hypothesis that scurvy in the guinea-pig is not due to the absence or deficiency of a certain factor in the diet but to the absorption of toxic products of bacterial activity brought about by the long retention of the faeces in the caecum caused by the unfavourable physical consistency of the diet. In his experiments he adopted the procedure of feeding the guinea-pigs with different sugars in order to induce an acidophilic bacterial flora instead of the usual putrefactive one and thus to obviate the possibility of the animals absorbing the toxic products of putrefaction which in his opinion are responsible for the production of scurvy in guinea-pigs. He claims to have achieved quite a decided success by adding lactose to the animals' diet: with other carbohydrates a marked beneficial effect was also observed. In this investigation Pitz again employed the same technique as was used by him previously in collaboration with McCollum, giving the guinea-pigs rolled oats and fresh whole milk *ad lib.* as a basal diet. We are therefore again unable to interpret Pitz's results in the same way as he does. Moreover we had records of some experiments at the time of the appearance of Pitz's paper which did not bear out that author's observations. In connection with another investigation we had the opportunity of feeding guinea pigs on cane sugar, fructose, and a syrup obtained after the fructose has been crystallised out from the products of the hydrolysis of inulin for which we are indebted to Prof. J. C. Irvine. These sugars, in the quantities we used, did not show the slightest antiscorbutic potency. Since the appearance of Pitz's publication we have also tested lactose, using high quantities and employing our technique, and as was expected no antiscorbutic activity was found to be possessed by the sample of the lactose used by us. We therefore consider it of interest to publish the record of our experiments.

EXPERIMENTAL.

In testing the antiscorbutic potency of a substance the following technique is employed in our laboratory. Guinea-pigs weighing from 250 g. to 450 g. are put on a basal diet of crushed oats and bran (one volume of oats to two volumes of bran) and a daily ration of 50 c.c. of milk autoclaved for one hour at 120°. On this diet the animal commences to drop in weight after about fourteen days and usually dies of scurvy within thirty days. In our experience no animal has ever yet failed to die of scurvy on such a diet. The substance to be tested is administered in daily rations *per os* or in some cases it is dissolved in milk which is quantitatively consumed by the animal. Fig. 1 represents the weight curves of guinea-pigs which received the scorbutic diet described above and 1 g. daily of Schering's fructose and of the uncrystallisable residues from the preparation of fructose from inulin respectively. Both these sugars were dissolved in the milk and were fully consumed by the animals until the last few days when, owing to their grave condition, the guinea-pigs were unable to consume much food. These animals died twenty-eight and thirty-four days after the commencement of the experiment and the post mortem examination disclosed a very acute condition of scurvy.

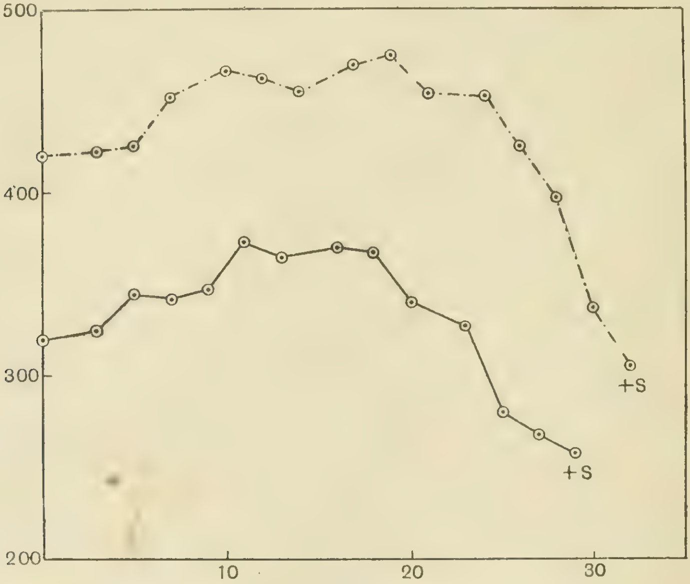

Fig. 1

⊙-.-⊙-.⊙-.-⊙ Schering's Fructose.

⊙——⊙——⊙ uncrystallisable residue from the preparation of fructose from inulin.

We have carried out no special experiments with cane sugar, but in the course of our work on scurvy we have on numerous occasions used generous doses of cane sugar for sweetening purposes without noticing the slightest beneficial effect, and we are convinced that it is scorbutically inactive.

Lactose was tested on three guinea-pigs receiving a basal diet of oats and bran and 50 c.c. of autoclaved milk. As in Pitz's experiments high doses were given. Two guinea-pigs received 6 g. of lactose and one 3 g. per diem in their milk. As will be seen from Table I about four-fifths of this was consumed daily. All the pigs died of scurvy within thirty days so that the onset was not even delayed. Fig. 2 shows the weight curves of the animals in question, which are typical weight curves of guinea-pigs kept on a scorbutic diet of oats, bran and autoclaved milk. A few days before their death the animals lost their inclination for food. In order to insure high consumption of lactose even at that stage the oats and bran were also mixed with lactose as well as the autoclaved milk.

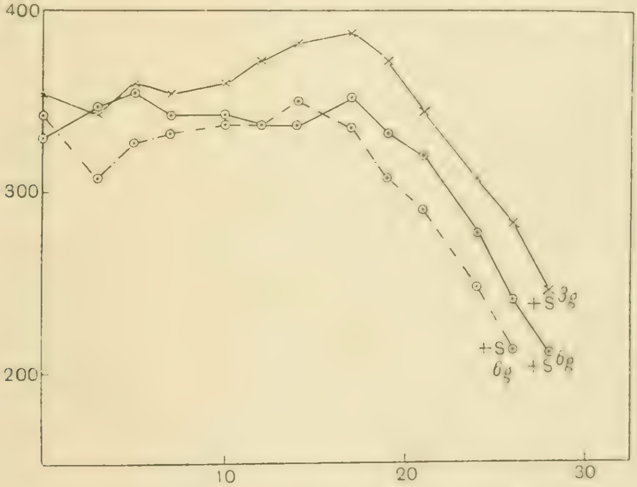

Fig. 2

It is evident from the above experiments that the sugars tested afford no protection whatsoever against scurvy, and the data tend further to strengthen our belief that any amelioration observed by McCollum and Pitz in their experimental animals was due to the enhanced consumption of the raw milk and not to the antiscorbutic potency of the substances tested. As long as these observers continue to employ their present technique we consider that their results are open to criticism.

Table I.

	Pig No. 269 (3 g. lactose)		Pig No. 270 (6 g. lactose)		Pig No. 271 (6 g. lactose)	
Days	Milk	Oats and bran	Milk	Oats and bran	Milk	Oats and bran
1	40 c.c.	19 g.	38 c.c.	24 g.	35 c.c.	10 g.
2	40	14	40	31	40	25
3	40	31	45	29	45	24
4	35	16	35	21	40	21
5	50	18	27	25	35	19
6	45	18	40	26	35	21
7	40	17	40	27	45	19
8	40	16	45	26	45	18
9	44	16	45	23	50	21
10	40	14	35	21	40	26
11	40	9	10	26	45	24
12	10	14	25	28	45	22
13	30	16	40	25	40	21
14	45	14	35	23	45	19
15	40	11	35	24	30	19
16	30	16	40	18	45	21
17	50	14	45	18	40	16
18	45	6	30	8	40	11
19	30	6	30	10	40	11
20	45	4	30	6	30	6
21	35	6	25	12	30	1
22	30	3	25	6	25	0
23	35	7	15	7	35	0
24	25	5	20	5	28	5
25	20	5	15	5	40	5
26	10	5	10	2	10	3
27	12	1	—	—	6	2
28	0	1	—	—	10	3
29	10	1	—	—	0	2
30	—	—	—	—	0	0

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XXIV. ON THE IMPORTANCE OF GEOLOGICAL FACTORS IN SEWAGE PURIFICATION.

By EVELYN ASHLEY COOPER and ALBERT EDWIN COOPER.

(*Received July 10th, 1918.*)

A great deal of work both from the scientific and the practical points of view has been carried out in connection with the biochemistry of sewage-purification by Letts and Adeney and other investigators for the Royal Commission on Sewage Disposal [1908, p. 5].

It is unnecessary to summarise the results of their work fully, but it may be briefly said that the investigations were concerned first, with studying the nature and course of the complex biochemical processes that go on when organic matter is decomposed by bacterial action, and the influence of various physical conditions thereon, and second, with elaborating and standardising the dissolved oxygen absorption test, commonly called the Rideal-Stewart test, in order to make this test of value in forming an opinion of the quality of a sewage effluent.

The majority of biochemists are now convinced that, although this test takes five days to complete when properly carried out, yet it yields most valuable results; and, as the bacterial processes involved are natural processes going on in river and other waters, it is to be preferred to the various other determinations often used in the standardisation of effluents, *e.g.* albuminoid ammonia, organic nitrogen, oxygen absorbed from permanganate, which are purely arbitrary and artificial and have no relation to the biological aspect of the question. The dissolved oxygen absorption test consists in measuring the amount of oxygen used up in a given period in the bacterial oxidation of the organic matter contained in the sewage effluent. According to Adeney this measures the amount of readily oxidisable carbonaceous matter present in the effluent, very little or no ammonia being oxidised during the five days' period usually given. As it is the quickly fermentable organic matter that will seriously pollute and deoxygenate a river-water, the value of this test is at once evident.

In standardising the dissolved oxygen absorption test the influence of various factors, *e.g.*, time, temperature, dilution, was investigated by the Commissioners [1913, p. 93], and it was recommended that the test should be carried out for five days at 18° C. The period of five days was chosen, because it appeared that sometimes there was an initial inhibition of the bacterial processes, so that low results might be obtained if shorter periods were employed, and the test would then be too lenient. The standard temperature of 18° C. was selected because it was found to be unusual for river waters in this country to exceed it.

The dilution chosen was 1 in 5, as this was a convenient dilution, and it was found that up to 1 in 5, dilution had little effect on the rate of absorption of dissolved oxygen. The method of analysis is fully given.

Although this test has been worked out in some detail the question as to whether the rate of biological oxidation of the organic matter is affected by the geological source and mineralogical constituents of the water used for dilution of the effluent has not apparently been sufficiently studied. The Commissioners [1913, p. 93] recommend that good tap-water should be employed, but it is obviously difficult to define this expression. In an addendum to another report [1908, p. 433] however it is pointed out that the results may depend on the nature of the diluting water, but examples are not given, except in the case of sea-water, which, although it has no measurable effect on the carbonaceous oxidation and production of nitrite from ammonia, yet was found to prevent nitrification most effectively. Fowler [1912] also states that in the case of a sewage effluent discharged into a river-water, nitrification was prevented by calcium chloride that was discharged into the same river from an ammonia-soda factory.

The importance of geological factors was suggested by some accidental observations at this laboratory. There being two separate water-supplies laid on, the question arose as to which should be employed for dilution of the effluents in carrying out the dissolved oxygen absorption test. One water was hard and calcareous, pumped up from the upper chalk, covered in the particular district by a considerable thickness of tertiary strata (Reading beds, London Clay and Bagshot Sands). The other water was a soft ferruginous upland surface water from the gravels and underlying upper Bagshot Sands, being thrown out by the clayey Bracklesham beds underneath.

The influence of various waters on the rate of dissolved oxygen absorption by sewage effluents.

In the experiments detailed below effluents were diluted with different waters, distilled water, tap waters, and stream waters. The samples of the latter were always taken from the streams into which the effluents were being discharged. For each set of experiments the conditions were kept constant throughout: dilution, temperature, volume, and period of incubation.

The experimental method consisted in incubating the aerated dilutions of the effluents in well-stoppered bottles for five days at 18° C. and estimating the concentration of dissolved oxygen left at the end of this period. The initial concentration of oxygen was determined by a separate test, and by difference the amount of oxygen absorbed or used up in the oxidation of the organic matter could be calculated.

A large number of experiments on these lines have been carried out over a period of a year. In order to economise space, however, only a few typical results are tabulated in this paper.

The main points emerging from the work are as follows.

I. Practical.

The Standardisation of Sewage Effluents.

1. The dissolved oxygen absorption figure (five days) for a sewage effluent diluted with hard tap-water (derived from the chalk) was only from one-twentieth to one-half the figure obtained for the same effluent diluted with distilled water, or stream water taken from the stream into which the effluent was discharged.

The following results illustrate this:

Dissolved oxygen absorbed by 100,000 parts of effluent.
Dilution made with

(1) Distilled water	(2) Hard water	(3) Stream water
2·15	0·45	—
1·25	0·17	0·45
8·1	0·46	4·2

2. The dissolved oxygen absorption figure (five days) for an effluent may be considerably lower when the effluent is diluted with stream water than when diluted with distilled water.

Dissolved oxygen absorbed by 100,000 parts of effluent.
Dilution made with

(1) Distilled water	(2) Stream water
1·25	0·45
2·36	1·20
8·1	4·2

In examples 1 and 2 the stream water was taken from a stream flowing over the Hythe and the Folkestone beds, and in example 3, from a river in the Bagshot Sands, receiving hard surface drainage water and sewage from a town.

3. The dissolved oxygen absorption figure (five days) of an effluent was somewhat lower, about 20 %, when dilution was made with a ferruginous peaty tap-water (upland surface supply) from gravel and upper Bagshot Sands than when made with distilled water.

4. The biological oxidation of sewage was almost entirely stopped for forty-eight hours by dilution with a very ferruginous water from the Folkestone beds (iron content of water: 2·5 parts per 100,000). No inhibition, however, at all was noticed after five days. There was no initial inhibition when the iron had been previously removed from the water by means of a Candy-filter.

The following results illustrate this:

Dissolved oxygen absorbed by 100,000 parts of effluent.

	48 hours	5 days
Dilution with distilled water	0·92	2·16
,, ,, ferruginous water	0·08	2·10
,, ,, filtered ferruginous water	0·92	2·06

The addition of soil taken from the ferruginous strata (iron content: 10 %) near the junction of the Hythe and Folkestone beds also inhibited the biological oxidation of sewage.

It is evident therefore that the results obtained with waters of different geological source are most divergent, and it is essential for the routine examination of sewage effluents that a fixed standard be adopted; otherwise the results may vary considerably according to the laboratory in which the effluents are examined and the tap-water employed. At first it might be thought that distilled water should always be used as a diluting medium in the standardisation of effluents, because it is independent of geological conditions and the only factors involved are the organic matter and mineralogical constituents of the sewage itself. The chief purpose however of standardising a sewage effluent is to ascertain whether the effluent is likely to exert a serious polluting effect on the stream or river into which it is being discharged. It is therefore necessary that the experimental conditions should approximate as closely as possible to the natural conditions. It seems reasonable therefore to use the river or stream water as the diluting medium, the sample of water being of course collected well above the point at which the effluent is discharged. It may be expected that different results will be obtained by using different river waters, but if for each effluent to be examined the diluting water is always taken from the river or stream into which the particular effluent enters, consistent results will be obtained, and the test will be carried out under well standardised conditions. If the river water is taken as the standard diluting medium, the use of hard tap-water or a strongly ferruginous water will clearly make the oxygen test far too lenient. On the other hand, the use of distilled water may render the test somewhat severe, and thus cause effluents to be condemned that would not in reality seriously pollute the water-way.

It is further suggested that in the routine examination of sewage effluents more attention should be given to the conditions of the river or stream below the point of discharge. As a check on the effluent test, it is desirable to determine regularly the dissolved oxygen absorption figure for the undiluted water collected from the river or stream well below the point of pollution, care being taken to ensure that the sample is taken where the effluent and river water are thoroughly mixed.

According to the report of the Sewage Commission [1913, p. 132] the dissolved oxygen absorption figures of waters unpolluted by sewage were always well below 0·4 part of oxygen per 100,000, and, if this limit were exceeded owing to the sewage pollution, conditions were likely to become offensive. It was recommended therefore that sewage purification should be sufficient to prevent an effluent from being of so bad a quality, that the Rideal-Stewart figure of the stream water below the point of pollution exceeded 0·4 part oxygen per 100,000. For average dilution this would mean that the Rideal-Stewart figure for an effluent should not exceed 2·0. This standard however is adaptable to conditions; if the stream were very small, a more stringent standard would have to be adopted; while in the case of a large river, considerable latitude could be given. During the past year many laboratory and field observations have been made in the case of numerous streams in Surrey and Hampshire, chiefly tributaries of the river Wey. As the result of these observations it has been possible to confirm the conclusion that for unpolluted waters the dissolved oxygen absorption figure does not exceed 0·4, and that where effluents are discharged into water-ways, in order to avoid nuisance, the effluents should be of such quality that the streams are kept well within the above limit.

2. Theoretical.

So far only the practical question of standardisation of effluents has been considered. We have also to enquire into the cause of the marked inhibitory effects described in the previous section. As already stated the hard tap-waters that were found to inhibit the biological oxidation of sewage were municipal water supplies obtained from borings in the chalk, which was covered in the particular districts by the Woolwich and Reading beds, London Clay and

Bagshot Sands. The waters contained a considerable amount of calcium bicarbonate and calcium sulphate (total hardness 24·0, permanent hardness 6·5, temporary hardness 17·5). Magnesium however was found to be absent. When the hard water was deprived of all calcium carbonate by boiling and filtering, it no longer exerted an inhibitory action on the processes of biological oxidation. This was taken as presumptive evidence that the inhibitory factor was calcium bicarbonate. If this were the case, it was thought that the addition of solid calcium carbonate should also inhibit the oxidation of a sewage effluent, as there should be sufficient carbon dioxide present to form soluble calcium bicarbonate. Preliminary experiments however showed that the biological oxidation was apparently accelerated by the addition of calcium carbonate. This was not due to impurities in the latter, as by itself it did not use up any dissolved oxygen. It was noticed however that when chalk was added to a sewage effluent, the initial titrations came out about 40 % lower, *i.e.*, there was an apparent loss of dissolved oxygen in the short time (thirty minutes) occupied in the estimations. It was ascertained by syphoning the liquid free from chalk before titration, that various chalks, *e.g.*, pure calcium carbonate, chalk from Upper, Middle and Lower Chalk Formations, and also chalk marls, did not adsorb dissolved oxygen from aqueous solution, and that accurate results were always obtained if care were taken to remove the chalk by syphoning off the supernatant liquid before estimating the concentrations of dissolved oxygen. It would appear that when the chalk was not removed before the titrations some of the dissolved oxygen was carried out of solution mechanically during the effervescence due to the CO_2 liberated by the action of the sulphuric acid added upon the chalk.

Subsequent experiments in which the precaution of removing the chalk before the commencement of the estimation of the dissolved oxygen was always taken showed that the addition of calcium carbonate had practically no effect on the rate of biological oxidation of sewage. The addition of magnesium carbonate had however a very pronounced inhibitory effect. There being no magnesium present in the hard waters used in the experiments described above, nothing definite can thus be said at present as to why these hard waters when added to sewage so strongly inhibited the processes of biological oxidation; and further work must be carried out on this subject.

In the case of ferruginous water from the Folkestone beds which was found to inhibit temporarily the oxidation processes, it is probable that the inhibitory factor was ferrous carbonate, which is known to be present in such waters, and to have a toxic effect on organisms.

MAIN CONCLUSIONS.

The work so far described is only of a preliminary nature, but is sufficiently advanced to show that in the dissolved oxygen absorption test for the standardisation of a sewage effluent, in order to obtain reliable results, it is important to employ, as a medium for diluting the sewage effluent, water collected from the river or stream into which the effluent is discharged, instead of the laboratory tap-water which is commonly used.

It is further recommended that, as a check in routine work, the river or stream water below the point of pollution be regularly tested, in order to ascertain if its dissolved oxygen absorption figure be below or above the limit of 0·4 part per 100,000.

It is too early yet to define the precise conditions and the extent to which geological factors may influence the processes of sewage purification. The results given in this paper have been obtained in connection with experimental work on sewage effluents in a restricted area, where the tap-water was yielded by sedimentary rocks of a simple kind—chalk, gravel, and sands. These results however suggest that, under different geological conditions, waters from other sedimentary rocks, and also waters associated with the detritus of eruptive rocks, where the active chemical constituents have been or are released may be expected to affect profoundly the biological processes of oxidation.

The practical biochemical work of this investigation has been carried out by one of us (E. A. C.) at the Hygienic Laboratory, School of Army Sanitation, Aldershot, while the geological aspect of the subject has been worked out by A. E. C.

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XXV. BIOCHEMICAL CHANGES DUE TO ENVIRONMENT.

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(*Received August 10th, 1918.*)

Observations on the influence of climate on the adaptation of alpine plants have so far been limited to the more or less obvious macroscopic or microscopic changes and their accompanying physiological effects on assimilation, transpiration and respiration. The most extensive investigations were those of Bonnier [1888, 1894] carried out in the experimental gardens in Paris and in the Montblanc range (1060–2800 m.), and those of Kerner [1891] who grew plants from seed in the botanical garden of Vienna and on the summit of the Blaser (2195 m.), near Innsbruck. The main conclusions to be drawn from this work were that the whole habit of the plants grown at higher altitudes became more stunted and dwarfed than in the plains, the development of the subterranean parts was relatively greater and the internal structure of both stem and leaf was modified, tending to lead to an increased power of assimilation. Of the principal factors of the alpine climate which gave rise to these changes the largest share is ascribed to the more intense illumination by day accompanied by low night temperatures and a drier atmosphere. Many observers as well as Kerner and Bonnier note the greater intensity of colour of alpine flowers and Bonnier attempted to confirm this subjective observation by comparing the colour tints of flowers at different altitudes in the mountains of Austria-Hungary by means of a graduated colour scale or "chromometer."

The rapid progress which has been made in the last few years in our knowledge of the chemistry of flower pigments makes it possible to attack these questions from a different point of view. It was first suggested by Miss Wheldale [1909] that a relationship exists between the anthocyanins and the flavones (or possibly xanthones). Whilst the theoretical assumptions of Miss Wheldale [1911] demanded hydrolytic and oxidative processes for the formation of anthocyanins from flavone glucosides, the experimental facts subsequently brought forward by Keeble, Armstrong and Jones [1913] led them to assume an interplay of reducing and oxidative enzymes. Combes [1913] was the first completely to contradict the oxidation theory and he showed that a reducing agent, presumably an enzyme, plays the fundamental rôle in the process. Everest [1914] confirmed this conclusion and was able to convert extracts of white and yellow flowers, probably containing flavone glucosides, without intermediate hydrolysis, into anthocyanins by making use of reducing agents, of which magnesium was the most suitable. Willstätter, in conjunction with Everest [1913] and with Mallison [1914] adduced definite proofs that all naturally occurring anthocyanins were glucosides of reduced flavones or flavonols, and furnished on hydrolysis the chromophoric component (anthocyanidin = reduced flavone or flavonol) and a carbohydrate (glucose or galactose)[1].

Shibata [1915] pointed out the universal distribution of flavone derivatives in the overground parts of plants, and in conjunction with Nagai and Kishida [1916] showed that alpine and tropical plants especially are rich in these substances. The Japanese investigators advanced the interesting suggestion that the presence in the peripheral tissue of flavone derivatives may possess an important physiological significance in absorbing the ultra-violet rays of sunlight and thus protecting the plant against the injurious action of these rays. They also demonstrated the fact that greatly diluted solutions of chemically pure flavone derivatives absorb the ultra-violet region of the iron arc light almost completely.

If the hypothesis of Shibata is correct one would expect that alpine plants grown at lower altitudes, in which ultra-violet light is known to be much less intense owing to its rapid absorption by the atmosphere, would not produce the flavone substances to the same extent as those grown in the Alps where the insolation is so much more intense.

In spite of the apparently adverse conditions (dampness of the atmosphere in winter and the absence of the protecting influence of a permanent layer of snow) alpine plants can be easily grown in this country and develop normally from the seedling to the flowering and fruiting stage, even in the neighbourhood of London where they are exposed to the influence of a smoke-laden atmosphere. One of the easiest to grow from seed is the well known Edelweiss (*Leontopodium alpinum*) and partly for this reason and partly because I had a considerable number of specimens collected by myself in the Alps available for comparison, I selected this plant for the experiments to be described.

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[1] For detailed discussion and full literature of this subject see the monographs of Everest [1915] and Wheldale [1916].

A large number of flowers from plants growing on my rock garden in a London suburb (80 m. above sea) were used in these experiments. They came from a plant collected six years ago on Mt. Colbricon, about 2000 m. above sea level (near San Martino di Castrozza), and from the seedlings of this plant, of which several generations had been raised. The plants are growing in different positions as regards aspect and soil, but in all cases care was taken that the soil contained a plentiful supply of calcium carbonate (mortar rubble), which is essential for the well-being of this and the majority of alpine plants.

The flowers which served for comparison with the lowland flowers were collected during a period extending over several years from various places in the central and eastern Alps (Switzerland, Tyrol, Dolomites), generally at a height of 2000 m., and had been preserved in the usual way[1].

The whole of the material was gathered approximately at the same stage of development of the plants (during middle and end of August) and was finally dried together for one month *in vacuo* over sulphuric acid.

The presence of flavone derivatives in the silvery hairs of the bracts of the Edelweiss inflorescence can easily be demonstrated by two reactions. An intense yellow coloration is at once produced on exposure of the flowers to ammonia vapour, a characteristic reaction of nearly all white flowers which was first made use of by Miss Wheldale. The second reaction is still more striking and may be demonstrated with as little as 0·2 g. of the flower bracts. The colourless, or slightly yellowish, alcoholic extract of the bracts gives on reduction with magnesium in acid solution (in the presence of mercury as a catalyst) an intense orange-red colour [Shibata, 1915].

Nature of the Chromogenic Substance. The isolation and identification of the chromogenic substance obviously demands a larger amount of material than was at my disposal. There can be little doubt that the substance belongs to the class of flavone derivatives, which occur in plants in the free state as well as in glucosidic combination. A very simple reaction makes it possible to decide whether the glucoside, the non-glucoside, or a mixture of the two is present in Edelweiss. In their work on flower pigments Willstätter and Everest [1913] devised the so-called "anthocyanidin" reaction which depends on the fact that the glucoside (anthocyanin) remains entirely in dilute sulphuric acid solution when shaken with amyl alcohol, whilst the non-glucoside (anthocyanidin) passes quantitatively into the amyl alcohol layer. When this alcoholic layer is shaken with sodium carbonate solution the pigment descends quantitatively into the aqueous layer. It is apparent that this reaction may be applied to the reduced coloured extracts, in which the flavone or its glucoside originally present, is reduced either to anthocyanidin or anthocyanin. It was found that plants collected in the Alps contained traces of the chromogenic substance in glucosidic combination, whilst the London grown plants contained only free flavones[1]. The reductions were carried out at a low temperature in order to avoid any acid hydrolysis of the glucoside, and the reduced solutions were diluted with water and freed from alcohol by shaking out with ether. On shaking with amyl alcohol the pigment passed quantitatively into it in the case of the homegrown plants, whilst a faint tint remained in the other cases even after repeated extraction with fresh amyl alcohol. The alcoholic solution showed the typical spectral band between λ 480 λ 520, allowing red and violet to pass. On shaking the amyl alcohol solution with sodium acetate the pigment remained in the alcohol, but it descended quantitatively into the aqueous layer on shaking with dilute sodium carbonate. The conclusion that the chromogen is present as a free flavone and not in glucosidic combination receives a certain amount of confirmation from the solubilities of the substance (insoluble in water, soluble in alcohol).

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[1] It had already been noticed by Shibata [1915] that flavones are not lost appreciably if the drying is done quickly, and that determinations can be made with any well preserved herbarium material, even if kept for a number of years.

Quantitative Relationship of Flavone Derivatives in Edelweiss grown in London and in the Alps. The colour produced by the reduction of the colourless alcoholic extract of Edelweiss obviously offers a method for comparing quantitatively the amounts of flavone derivatives formed in the plant under different environmental conditions. This may be carried out either by comparing the colour intensity of the various extracts *inter se* or with a standard solution. The finding of a suitable standard makes the latter procedure difficult. An attempt was made to use the colour produced on reducing alcoholic apigenin (1. 3. 4′-trihydroxyflavone)[2] solutions of known strength by means of magnesium. The colour of the reduced solution, although resembling that of the reduced Edelweiss extracts to the naked eye, was found on comparison in Duboscq's colorimeter to differ in its tint to such an extent as to make exact comparison impossible.

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[1] The white flowers of alpine saxifrages and of *Dryas octopetala* also contained free flavones only. The majority of white non-alpine flowers which I examined, on the other hand, contained practically the whole of their chromogen in the glucosidic combination.

[2] Apigenin was obtained by the hydrolysis of the glucoside apiin, which I prepared from parsley. I am indebted to Dr Barger for another sample of pure apiin, which gave identical results.

The complete extraction of the flavone at first offered some difficulties. Exhaustive extraction with alcohol in a Soxhlet's apparatus introduced variable amounts of greenish-yellow decomposition products of chlorophyll which could not be completely removed by shaking out with light petroleum. Although small in amount they were sufficient to influence the tint of the reduction colour. The following procedure was finally adopted. Quantities of 0·5 or 1·0 g. of the plant organs were repeatedly extracted with twenty parts of 90 per cent. alcohol at 70–75° for some minutes, until the decanted extract no longer gave any colour after reduction. At least six extractions were found to be necessary. Each extract was filtered through a small filter-paper (2 cm.) into a graduated test tube and 1 cc. conc. HCl was added to every 4 cc. of the extract. After the addition of a few drops of mercury the acid solution was reduced by magnesium powder, added gradually in small amounts until the maximum colour intensity was reached. After reduction the coloured extracts were mixed and made up to 50 or 100 cc. respectively with 90 per cent. alcohol.

It became at once obvious that the colour intensity of the reduced extract from the plants collected in the Alps was much stronger than that of the plants grown in the lowland, so much so that it became necessary to dilute it largely before comparison in the colorimeter. Estimations were made by means of Duboscq's and Hellige's colorimeters. Owing to the presence of small amounts of chlorophyll decomposition products, the colour of extract (2) could not be matched in Duboscq's apparatus, but the slight difference in tint did not interfere with the estimation in Hellige's wedge colorimeter. The results of many readings, in which the standard was set at different heights, were as follows:

Alpine Flowers	Flowers from London Plants			
	(1)	(2)	(3)	(4)
100	25·2	26·0	28·1	21·6

It will be seen therefore that the reduction colour, which serves as an index of the amount of flavone originally present, reaches on the average in London grown plants only 25 per cent. of that produced in the plants grown at higher altitudes.

Distribution of Flavone derivatives in Edelweiss plants. Whilst the flower-heads as a whole contained a large amount of flavone, it was found that the stems of plants collected in the Alps contained much less and the stems of London grown plants were quite devoid of chromogenic substance. On the other hand the fruit (achene with its pappus) is extremely rich in it. Extracts of fruits and the corresponding flower bracts were compared quantitatively with the result that the reduction colour of the flower bracts was found to be a little more than half (54 per cent.) of that of the fruits. It seems therefore that the flavone derivatives are stored up in the seed and in accordance with this it was found that the inflorescence gathered in November had lost with its fruit the whole of its chromogenic substance. Vegetative leaves as well as roots of plants examined in December, were also devoid of chromogen.

The Flavone Contents of Edelweiss as a Biological Factor. The distinguishing feature of this plant, especially of its inflorescence, consists in its covering by long woolly hairs which give rise to the idea of whiteness expressed in its name. As an explanation for the biological significance of this pubescence it has been assumed that the air entangled between the matted hairs checks excessive transpiration and helps the plant to exist under very dry or desert conditions. The fact that under the influence of the moist English climate the plant continues through many generations to produce this characteristic feature is to some extent against this explanation, and together with the results of the above described observations tends to lend support to the suggestion of Shibata. The growing season of the alpine plants coincides with the maximum intensity of chemical sunlight in the year and the necessity of some protective mechanism of the living protoplasm against the injurious action of ultra-violet light is apparent. The flavones dissolved in the cell sap may well exert this protective action, as they do when examined in ordinary solution. The same plants grown in London under conditions in which the insolation is much less intense, apparently adjust themselves to the altered conditions by producing the protective substances in smaller amounts. In this connection it is interesting to note the behaviour of some other white alpine flowers which I had occasion to examine in February, a month in which the amount of ultra-violet light is extremely low. Extracts of the petals of *Saxifraga burseriana* and of *S. Boydii alba* (burseriana × aretioides) which flowered in my garden in the first half of February, gave practically no colour on reduction. On the other hand the white flowers of saxifrages (*S. cotyledon*, *S. longifolia*, *S. lingulata*, *S. Wallaceii*), examined later in the season, contained flavones. The petals of *Dryas octopetala* and *Campanula pusilla alba*, also gave intense reduction colours when examined in May and July respectively.

SUMMARY.

The inflorescence of Edelweiss (*Leontopodium alpinum*) contains a chromogenic substance, probably a flavone, which is not in glucosidic combination. Plants grown in London at an altitude of 80 m. contain only one fourth of the amount found in plants collected in the Alps at an average altitude of 2000 m. These facts illustrate the biochemical adaptation of alpine plants to changed environment and speak in favour of the assumption that the biological significance of flavones in the plant consists in their protective action against the injurious influence of ultra-violet light.

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XXVI. ON AMINO-ACIDS.

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(Report to the Medical Research Committee.)

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The following paper contains results of investigations on the separation, preparation and properties of various amino-acids and their derivatives. Some new methods have been made use of which promise to be of considerable value and have already led to the isolation of a new amino-acid and peptide. The complete working out of the experimental details will take a considerable time, but as certain of the results already obtained are of a definite character and the methods appear to have a wide applicability, it appears desirable to give an account of the work at the present time, leaving many obvious extensions as subjects for further study. The material here presented is divided into three main portions, which will be considered separately:

I. The extraction of amino-acids by means of partially miscible solvents.

II. Hydroxyglutamic acid, a new hydroxyamino-acid of the dicarboxylic acid group.

III. A new peptide, isoleucylvaline anhydride, obtained by the acid hydrolysis of caseinogen.

I. THE EXTRACTION OF AMINO-ACIDS BY MEANS OF PARTIALLY MISCIBLE SOLVENTS.

The writer has long sought some means of extracting amino-acids from their aqueous solutions. Experiments were made with a variety of acid or basic solvents, in the hope of extracting basic or acidic amino-acids, but without success. Later experiments were made in which attempts were made to extract the strongly acid methylene amino-acid derivatives obtained by the action of formaldehyde, but the results were unsatisfactory. Recently, however, observations were made which led to a surprisingly simple method of extracting practically quantitatively many amino-acids from their aqueous solutions. For a particular purpose, the writer was anxious to obtain some optically pure proline, and as the ester method invariably gives a largely racemised product, it was decided to attempt the direct extraction of proline from the products of hydrolysis of caseinogen by means of alcohols. The probability of success seemed small, since Fischer and Abderhalden [1904], in writing of the separation of proline from other amino-acids by means of alcohol, strongly emphasise the necessity of a previous distillation of the amino-acid esters. However, on hydrolysing gelatin with sulphuric acid and subsequently removing the latter as barium sulphate and then extracting the resulting concentrated solution of amino-acids with amyl alcohol in a continuous extractor, it was found that the proline was readily extracted. Soon after the extraction was started, the surprising observation was made that large quantities of solid amino-acids separated from the alcoholic extract and this seemed the more curious since they are practically insoluble in ordinary amyl alcohol. The phenomenon seemed sufficiently interesting to warrant further investigation and similar experiments were made with other alcohols. Propyl, butyl and isobutyl alcohols and various mixtures were used and it was found that butyl alcohol was the most satisfactory. This substance is now obtainable at nominal cost, owing to its large production in the fermentation process of acetone manufacture. Its boiling point, lower than that of iso-amyl alcohol, is a practical convenience and its rate of extraction is as good as that of any of the others.

On extracting amino-acid solutions from caseinogen and other proteins with butyl alcohol, it was found that the proline and practically the whole of the monamino-acids were extracted, together with some peptide anhydrides, which will be referred to later. The strongly ionised dibasic and dicarboxylic acids were left behind practically quantitatively. The only "neutral" monamino-acids detected in the extracted material have been minute traces of serine and in the case of amino-acids from gelatin the complete extraction of glycine seemed more difficult than that of the other amino-acids. In general, the extraction is surprisingly rapid and with the simple apparatus employed the extraction of two or three hundred grams of mixed amino-acids is complete in 36 hours. The bulk of the monamino-acids separate out in the extraction flask as a cream-coloured granular powder. It is convenient to filter off the monamino-acids occasionally during the extraction in order to avoid bumping or local overheating, using the mother liquor for continuing the extraction. The solid monamino-acids are washed first with a little butyl alcohol, then with a little ether, which removes traces of pigment and water. The butyl alcohol mother liquor contains the whole of the proline, together with small amounts of amino-acids and peptide anhydrides, which can be largely separated through their sparing solubility in alcohol or water.

On examining the residue of non-extracted amino-acids, it was found to contain practically the whole of the diamino-acids, as judged by quantitative precipitation with phosphotungstic acid, and also the dibasic acids, aspartic acid, glutamic acid and a new dibasic acid to be described later. Larger yields of the dibasic acids were obtained from caseinogen than have previously been obtained by other methods. None of the dibasic acids could be detected among the extracted monamino-acids, so that it is clear that for practical purposes, they may be regarded as quantitatively separated from the latter.

It is thus seen, that by the use of butyl alcohol as a solvent, the products of hydrolysis of a protein may be readily separated almost completely into the following five groups:

(1) Monamino-acids, both aliphatic and aromatic, insoluble in alcohol but extracted by butyl alcohol.

(2) Proline, soluble in alcohol and extracted by butyl alcohol.

(3) Peptide anhydrides (diketopiperazines) extracted by butyl alcohol, but separated from (2) by sparing solubility in alcohol or water.

(4) Dicarboxylic acids, not extracted by butyl alcohol.

(5) Diamino-acids, not extracted by butyl alcohol, but separable from (4) by phosphotungstic acid and other means.

Those who have had much experience in the separation of amino-acid mixtures will appreciate the following points. (*a*) The groups are composed of chemically similar individuals. (*b*) Practically the whole of the products of the hydrolysis of an amino-acid may be divided into the five groups without serious loss and each can be readily obtained in solid form, aliquot parts of which may be used in the search for individual acids. (*c*) No indications of any racemisation have been observed. All the amino-acids thus far separated possessed their full optical rotation, thus limiting the number of isomers in the various mixtures and permitting greater use of the polarimeter for identification purposes. The absence of racemisation has an obvious bearing on the use of the method for purposes of preparing material for bacterial and animal metabolism experiments. (*d*) Materially higher yields of many amino-acids may be obtained from proteins than by existing methods, thus permitting a more nearly quantitative analysis of the proteins themselves.

The apparent paradox of the extraction from aqueous solutions of monamino-acids, which are essentially insoluble in all alcohols, by means of butyl alcohol or similar compounds requires some reference. It soon became clear that the presence of water was a conditioning factor and that the passage of a certain proportion of water from the fluid undergoing extraction to the butyl alcohol medium, was essential. It was found that a concentration of amino-acids rapidly extractable by butyl alcohol yielded almost no amino-acids in the extract, if the aqueous phase contained an excess of salts such as calcium chloride. The obvious inference was that the passage of water from the aqueous to the butyl alcohol phase was impeded with corresponding reduction in the rate of extraction. Butyl alcohol at room temperature is miscible with about twelve parts of water, but I know of no experiments on the relative distribution of water and butyl alcohol when shaken together in excess. It can hardly be doubted that the successful extraction of monamino-acids from aqueous solutions by butyl alcohol is dependent both on the limited passage of butyl alcohol into the aqueous layer and of water into the alcohol layer. The conditions empirically found in the present case seem excellent for the purpose in view, but it would seem well worth while to consider the possibility of a much more extensive use of the present principle with other extractable substances and solvents. So far as I am aware, no systematic investigation of the possibilities of extractions by partially miscible solvents has been undertaken, though the common method of extraction of aqueous solutions with ether is in reality an extraction with ether plus water, and various substances, *e.g.* hydantoins, can be quantitatively extracted in this way from aqueous solutions, although they are almost insoluble in pure ether, and readily soluble in water. It can hardly be doubted that the use of butyl alcohol and similar solvents, will be found of value for many other purposes than the one referred to. It is not unlikely that certain sensitive substances may be selectively extractable from tissue extracts with a minimum of alteration, especially if the extraction be carried out under reduced pressure and hence at a reduced temperature. Tryptophan, formed from caseinogen by tryptic digestion, is readily extracted practically quantitatively in an almost pure condition after a single preliminary purification with mercuric sulphate. Another obvious application of the method is in the study of the products of the hydrolysis of proteins by enzymes.

EXPERIMENTAL.

The earlier experiments in which solvents other than butyl alcohol were used, will not be described, since the latter solvent appears preferable. Hydrolysed gelatin was used in many preliminary experiments, but as the results with caseinogen are rather more complete, they alone will be considered.

Hydrolysis. Purified caseinogen varying in amounts from 100–400 g. was hydrolysed by boiling for 12 to 16 hours, with five to six times its weight of diluted sulphuric acid prepared by mixing the concentrated acid with three volumes of water. Hydrolysis under these conditions is still incomplete, judged by the subsequent separation of peptide anhydrides in significant amount. After the heating was ended, the solution was diluted with water and the sulphuric acid quantitatively removed with barium hydroxide in the usual way. The filtrate, which still reacts acid to litmus but not to congo-red, was concentrated moderately and about 2 per cent. of crude tyrosine allowed to crystallise out. The filtrate was again concentrated and made approximately neutral to litmus by the further addition of barium hydroxide. The optimum reaction has still to be determined and probably varies with different proteins. In the case of gelatin feebly acid and alkaline reactions obtained by adding acetic acid or ammonia were successively tried, but this is not necessary with caseinogen and it is doubtful if it is ever desirable.

Extraction. The approximately neutral solution of amino-acids prepared as above described was concentrated on the water-bath until crude leucine began to separate and then the whole mixture was transferred whilst still warm to the extraction apparatus. Usually an apparatus of the simple Kutscher-Steudel type was employed, although others proved equally satisfactory. The amino-acids from 100 g. of caseinogen may be conveniently extracted in an apparatus holding about 350 cc. It is preferable to arrange conditions so that the aqueous layer occupies three-quarters or five-sixths of the available space and the volume of supernatant butyl alcohol is relatively small. It has been found convenient to substitute a rather larger extraction flask made of tough glass of the Jena type for the smaller flask usually employed. Rubber connections can be employed, although they are attacked by the hot alcohol to a considerable extent and their exposed surface should be reduced to a minimum. The extraction flask is heated over a sand-bath, preferably with rather high sides, so as to reduce condensation in the flask, and the flame is adjusted so that a reasonably rapid flow of alcohol returns into the flask. When using a 300–400 cc. or smaller apparatus, the aqueous solution soon attains a temperature of from 60°–80° and neither cooling nor heating is required, but when larger volumes of fluid are being extracted, it is a good plan to warm it to 75 or 80° by means of a water-bath.

Soon after the extraction has begun, a separation of cream-coloured granular particles of amino-acids is observed in the extraction flask and the amount steadily increases. After extraction has continued during a working day, it will be found convenient to allow the extract to stand overnight and to filter off the separated amino-acids on a Buchner funnel, washing them with a little butyl alcohol and finally with a little ether. The combined alcoholic filtrates which are light brown in colour are used for continuing the extraction on the second day. Risk of breakage due to the accumulation of solid amino-acids on the bottom of the flask is thus avoided and it is possible to judge the completeness of the extraction by observing the increment of solid amino-acids on continued heating. When after several hours' extraction no significant additional amount of solid matter separates out of the butyl alcohol solution, the operation is regarded as finished. With the apparatus employed and extracting 1 to 400 g. of mixed amino-acids, the operation usually took about 36 hours; the time varying naturally with the rate at which the butyl alcohol is boiled.

When the extraction is over, the aqueous solution is reserved for examination for bases and dicarboxylic acids, as will be described later. The butyl alcohol extract, containing the proline and some peptide anhydrides, is filtered from the separated amino-acids, after standing at room temperature for some hours, while the monamino-acids, filtered and washed as previously described, are dried and weighed.

Butyl alcohol extract. Separation of proline, etc. The filtered extract is evaporated to dryness in a distillation flask under reduced pressure and the sticky brownish residue boiled with about ten parts of absolute alcohol. In the case of caseinogen at least, the whole of the residue is soluble in ethyl alcohol, indicating the absence of any considerable quantity of monamino-acids other than proline. On allowing the alcoholic solution to stand overnight, crystallisation occurs with separation of a small quantity, averaging 2 per cent. of the caseinogen, of an impure mixture chiefly composed of peptide anhydrides with traces of monamino-acids. This product was filtered off, washed with alcohol and examined later. The alcoholic filtrate was then evaporated under reduced pressure, dissolved in hot water, boiled with charcoal, filtered and concentrated to syrup.

A further crystallisation of peptide anhydrides occurs on standing, averaging 2 to 3 per cent. of the caseinogen taken, and this is filtered off and examined together with the similar product obtained above. The total yield of peptide anhydrides in different experiments was as follows: 2·7, 2·2, 3·2, 5·0 and 5·7. The larger yields were obtained from experiments with the larger amounts of caseinogen.

The clear aqueous solution after separation of the two fractions just described contains the whole of the proline. It is already much purer than might be anticipated from its method of preparation and compares favourably with the alcohol-extracted product obtained by the ester method.

It appears that the actual proline content can be closely estimated by means of van Slyke's methods for differentiating between amino and imino nitrogen, for a characteristic derivative of proline was isolated in amount corresponding closely to that indicated by analysis. It was found that no purification was effected by converting the proline into its copper salt for the whole of the product was soluble in methyl alcohol. It will be convenient to give the yields of proline as indicated by van Slyke's differential analysis and then to refer to a new derivative of proline which seems useful for purposes of characterisation.

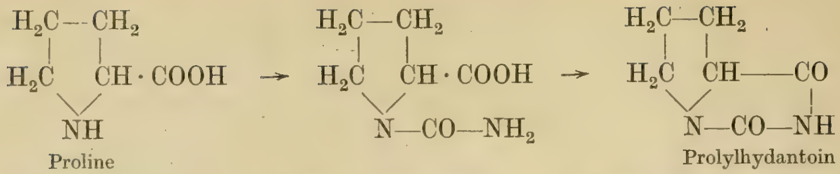
Caseinogen analysed	Nitrogen of Proline Fraction		Percentage Proline calculated	% NH
	Total	Non-Amino		
150 g.	1·70 g.	1·34	7·3	[illegible]
64	0·683	0·557	7·1	[illegible]
50	0·611	0·521	8·5	[illegible]
18	0·224	0·184	8·2	[illegible]
232	2·94	2·45	8·7	[illegible]

The average of the above determinations is close to 8·0 per cent. proline and evidence other than analytical will be presented to show that no considerable amount of other substance than proline is contained in the above estimate. It is of interest to compare the above figures for the proline content of caseinogen with that obtained by other workers. Van Slyke [1911], using his analytical methods on amino-acids separated by the ester method, obtained 6·70 per cent., while his analysis of caseinogen by direct hydrolysis indicated that 7·13 per cent. of the total nitrogen of caseinogen was in the non-amino form. If the whole of this were due to proline, ignoring the small amount of hydroxyproline it would be equivalent to 9·2 per cent. proline. Abderhalden obtained only 3·1 per cent. of proline by the ester method, while Foreman in unpublished experiments is quoted by Plimmer [1917] as having obtained 7·6 per cent., a figure in close agreement with that found by me.

By the direct extraction method just described, there is no evidence of any racemisation of the proline as judged by the solubility of copper salts in methyl alcohol and by the preparation of other derivatives, but on the other hand, the impurities which accompany the proline appear to be fairly strongly dextro-rotatory, so that a direct polarimetric estimation of proline is not possible. The observed rotations correspond to about three-fourths of the proline, believed to be actually present.

Preparation of l-*prolylhydantoin.* The preparation of uramido-acids and the hydantoins derived from them by the action of mineral acids has not been made as much use of for the identification and separation of amino-acids as the method deserves. In many cases the products are more easily purified than the more generally known phenyl derivatives, obtained by the use of phenyl isocyanate, on account of well-marked solubility differences. Proline affords a good example of the utility of the method. On warming crude proline on the water bath with a moderate excess of potassium cyanate, it is converted smoothly into the uramido-acid, and the same is true of any contaminating amino-acids. On liberating the uramido-acids by making the solution just decidedly acid to congo-red and extracting with ether for a few hours in a continuous extractor, practically none of the proline compound is extracted, but the whole of any leucine, isoleucine, valine, alanine, phenylalanine and tyrosine derivatives are removed. On heating the extracted aqueous solution with 10-20 per cent. sulphuric or hydrochloric acid for an hour and then re-extracting with ether, prolylhydantoin is obtained in excellent yield and possesses convenient properties for purposes of identification. The following gives details of a typical experiment.

Crude *l*-proline (5 g.), containing as judged by van Slyke's analytical methods about 16 per cent. of impurity, was evaporated on the water bath with potassium cyanate (5 g.) and water (50 cc.). On taking up with water, acidifying to congo-red with sulphuric acid and extracting with ether for seven hours, 0·55 g. of uramido-acids, chiefly derived from valine and leucine, were obtained. The extracted aqueous solution was then heated for 1-2 hours on the water-bath after the addition of one-fifth volume of 50 per cent. sulphuric acid and then again extracted with ether for 12 hours. On evaporation of the ether 4·3 g. prolylhydantoin was obtained in the form of transparent shining thick prismatic needles. The substance is very easily soluble in hot water and readily crystallises from the concentrated solution. The yield of pure products amounted to about 85 per cent. of the theoretical amount, after allowing for the known impurity in the crude proline.



Proline

Prolylhydantoin

l-Prolylhydantoin has a high optical rotation, as shown by the following observations on two different preparations:

(1) $c = 2{\cdot}0$, $l = 1{\cdot}0$, $\alpha = -4{\cdot}77°$ hence $[\alpha]_D^{20°} = -238{\cdot}5°$,

(2) $c = 2{\cdot}0$, $l = 1{\cdot}0$, $\alpha = -4{\cdot}64°$ hence $[\alpha]_D^{20°} = -232{\cdot}0°$.

On analysis, the two specimens were found to contain 19·80 and 19·95 per cent. nitrogen (Kjeldahl) compared with a calculated value of 20·0 per cent. *l*-Prolylhydantoin, after recrystallisation from water, melts at 165–167°. When prepared from partially racemised proline obtained by the ester method, the melting point is materially lower, *e.g.* 153–156°.

It is not without interest to note that *l*-prolylhydantoin is racemised by alkali at room temperatures much less readily than most other hydantoins observed by the writer. A 1 per cent. solution in 0·4 *N* NaHO still possessed about one-third of its initial rotation at the end of a week. The writer has advanced the view that racemisation of hydantoins by alkali at low temperatures is due to keto-enol tautomeric changes and as the CH group adjacent to the CO in prolylhydantoin, unlike the other hydantoins examined, forms part of the cyclic structure, it is not improbable that this fact is related to its slower rate of racemisation.

It is believed that *l*-prolylhydantoin may serve as a useful derivative for the characterisation of proline in impure mixtures, when successively converted into uramido-acid and hydantoin. It has been already stated that under these conditions, contaminating alanine, valine, leucine, isoleucine, phenylalanine and tyrosine are removed as uramido-acids on extraction with ether. On the other hand, glycine, serine, proline, hydroxyproline, aspartic and glutamic acids, are not extracted by ether as uramido-acids, but are extracted after conversion into their hydantoins. The practical utility of these facts under various conditions, is sufficiently obvious and will be studied in further detail.

The monamino-acids extracted by butyl alcohol.

The amino-acids extracted by butyl alcohol other than proline, separate out as previously described as a cream-coloured granular deposit. They dissolve in water to give a clear light yellow solution and contain only insignificant traces or none of the diamino-acids or dibasic acids. The reaction of the aqueous solution is barely acid to litmus.

The yield of these monamino-acids from caseinogen is indicated as follows:

48 g. caseinogen gave 18 g. dry monamino-acids = 37·5 per cent.
150 ,, ,, ,, 51 ,, ,, ,, = 34·0 ,,
232 ,, ,, ,, 86 ,, ,, ,, = 37·3 ,,

The above figures do not include about 2–3 per cent. of tyrosine filtered off prior to extraction, so that the total yield is approximately 40 per cent. of the caseinogen taken. It is not unlikely that slightly higher figures might follow greater care in avoiding loss in the removal of sulphuric acid following hydrolysis. The amino-acids thus separated contain about 11·5 per cent. of nitrogen and are chiefly made up of alanine, valine, leucines and phenylalanine, with a little tyrosine. Hydroxyproline and serine appear to be present, but the complete analysis of the mixture awaits further investigation. The product is in a most convenient form for application of the ester method, but it is believed probable that preliminary separations can be advantageously made. Apart from the traces of valine and leucine which accompany the crude proline, it is believed that the whole of these acids are contained in the fraction under consideration and most careful search by various methods has failed to detect them in the other fractions, provided the preliminary extraction with butyl alcohol has been adequate. Glycine, which appears to be absent or only present in traces in caseinogen, is only extracted quantitatively with difficulty, as experiments with gelatin showed. Traces of serine have also been detected among the unextracted amino-acids. A further report on the composition of this monamino fraction will be made at an early date.

Experiments on the rate of extraction by butyl alcohol of individual amino-acids from pure aqueous solution will also be made.

It is perhaps not out of place to refer to the possible use of such a readily obtainable dry, almost neutral, amino-acid mixture for furnishing a basis for nutrient media, with or without the addition of tryptophan. The fact that most of the amino-acids which furnish glucose in the diabetic organism are absent from this material suggests dietetic possibilities.

The amino-acids not extracted by butyl alcohol. Separation of aspartic, glutamic and other acids.

As has already been stated, the amino-acids which are not extracted by butyl alcohol from aqueous solution, are the strongly ionised diamino-acids and dicarboxylic acids. Quantitative experiments, in which the mixture was precipitated with phosphotungstic acid, showed that the whole of the diamino-acids could be recovered in this way, but the filtrate yielded less of the dicarboxylic acids than could be obtained by other methods, so that for purposes of quantitative analysis of proteins, it is decidedly preferable to use an aliquot portion for the examination of the bases and the rest for the dicarboxylic acids.

The diamino-acids. The precipitation with phosphotungstic acid and subsequent separation of histidine, arginine and lysine requires no comment other than the desirability of removing dissolved butyl alcohol from the solution by heating before precipitation, for the inhibitory action of alcohols on the precipitation is well known.

The dicarboxylic acids. The degree of purification effected by the removal of the mono-amino acids by extraction, permits a much more nearly quantitative separation of glutamic acid as hydrochloride than is usually the case. Precipitation moreover is usually more rapid and after removing the first crop of crystals, concentrating and re-saturating with hydrogen chloride in the usual way, the second crop of crystals is very small. It may be noted here that in the analysis of gelatin and other proteins containing much glycine, the precipitation of glycine hydrochloride along with the glutamic acid hydrochloride is possible on long standing in concentrated solution. Caseinogen gave the following yields of glutamic acid hydrochloride. In each case, the glutamic acid hydrochloride was filtered off on asbestos and well washed with cold hydrochloric acid. It was then dissolved in a small volume of water and again precipitated by saturation with hydrogen chloride. The only impurities to be feared were ammonium chloride and barium chloride and these were excluded by controlling the purity of the hydrochloride by analysis, using the Kjeldahl or van Slyke methods, after evaporation of the portion used for analysis with calcined magnesia. The values obtained by either method were all within 0·1 per cent. of the theoretical value of 7·65 per cent. of nitrogen.

I. 16 g. caseinogen gave 4·3 g. glutamic acid hydrochloride = 21·6 % glutamic acid.
II. 50 ,, ,, ,, 13·2 ,, ,, ,, ,, = 21·2 ,, ,, ,,
III. 150 ,, ,, ,, 36·7 ,, ,, ,, ,, = 19·6 ,, ,, ,,
IV. 232 ,, ,, ,, 42·6 ,, ,, ,, ,, = 14·7 ,, ,, ,,

The first three experiments quoted above gave reasonably concordant results indicating an average of about 21 per cent. of glutamic acid. The last experiment, IV, differed from the others in that previous precipitation of the diamino-acids with phosphotungstic acid was carried out and the filtrate was then freed from sulphuric and phosphotungstic acids by means of barium hydroxide. There was undoubtedly considerable loss connected with the handling of the bulky precipitates, and it seems reasonable to exclude this low result in computing the average. The proportion of glutamic acid in caseinogen, as estimated above, is materially higher than that previously recorded, with the exception of Foreman's estimations [1914]. Foreman obtained about 17 per cent. of glutamic acid as such, together with pyrollidone-carboxylic acid, determined analytically and presumably derived from glutamic acid, indicating a total yield of 21·8 per cent. glutamic acid. These figures are in excellent agreement with those now presented and are six to 12 per cent. higher than those previously recorded.

The next problem was the separation of aspartic acid from the filtrate from the glutamic acid hydrochloride. Various obvious methods were tried with unsatisfactory results, partly due to the presence of another dicarboxylic acid, to be described in the following section. Levene and van Slyke [1910] showed that aspartic acid was almost completely precipitated on boiling its aqueous solution with excess of lead oxide, the apparent solubility of the salt being about 1 in 4700. This fact was utilised successfully for the separation of aspartic acid as follows. The filtrate from the glutamic acid hydrochloride was concentrated under reduced pressure to remove as much of the hydrochloric acid as possible and the resulting syrup dissolved in about ten parts of hot water. It was then treated with calcium hydroxide and the calcium salts precipitated by alcohol as described by Foreman [1914]. The sticky deliquescent calcium salts after washing with alcohol were dissolved in water and the calcium removed exactly with oxalic acid. Freshly precipitated and well washed lead hydroxide was then added by degrees while the solution was kept boiling under a reflux condenser. The addition of lead hydroxide was continued until the reaction of the solution was distinctly alkaline to litmus and heating was then continued for a further 15 minutes. The mixture was then allowed to cool and stand overnight. The precipitate containing the aspartic acid and much lead chloride was filtered off and washed with cold water; the filtrate being reserved for the separation of the acid to be described in the following paper. The lead precipitate is somewhat difficult to decompose with hydrogen sulphide, so that it is more convenient to boil it with dilute sulphuric acid in moderate excess. Almost all the lead is thus removed and the filtrate contains aspartic, hydrochloric and sulphuric acids. The filtrate is then concentrated with excess of precipitated barium carbonate, filtered and made up to a convenient volume such as 1 cc. per gram of protein taken. A nitrogen estimation on a small aliquot part gave an indication of the possible aspartic acid concentration. A hot concentrated solution of normal copper acetate was then added in amount equal to 1·5 molecules per molecule of aspartic acid. On allowing the solution to stand for a couple of days in a cool place, the characteristic crystalline copper aspartate separated out almost completely and was filtered off, washed, dried and weighed.

In two experiments, 150 g. of caseinogen gave 9·7 and 11·6 g. of crystalline analytically pure copper aspartate. The salt, after drying in the air, contained more nearly 3 mols. of water than the more customary 4½, similar observations having been made by Curtius and Koch and by Skraup. The anhydrous salt dried at 130° contained 32·6 per cent. copper and 7·2 per cent. nitrogen, compared with calculated values 32·7 and 7·2 respectively.

The yields of crystalline copper aspartate noted above correspond to 3·5 and 4·1 per cent. aspartic acid respectively, in caseinogen, and are two to three times as large as the figures previously recorded.

The extraction of tryptophan by means of butyl alcohol.

The hydrolysis of caseinogen by sulphuric acid completely destroys the tryptophan, as is well known, and none of it was encountered in the preceding separations of the products of acid hydrolysis. It appeared desirable to determine whether tryptophan if present in a solution could be extracted, and accordingly the following experiment was made.

Caseinogen (187 g.) in two litres of 1 per cent. sodium carbonate solution was digested for seven days with active pancreas extract. The solution was then treated as described by Hopkins and Cole [1902] and precipitated *once* with mercuric sulphate solution. The precipitate was decomposed with hydrogen sulphide, sulphuric acid removed exactly with barium hydroxide and the solution concentrated to about 30 cc. under diminished pressure. It was then extracted with butyl alcohol in a small extractor. A separation of tryptophan on the sides of the extraction flask was soon noted, and at the end of two hours' extraction 1·5 g. had separated out. The extraction was practically quantitative at the end of 12 hours and the residual aqueous solution gave only a doubtful reaction with Hopkins' sensitive glyoxylic acid reaction. The tryptophan crystallising from the butyl alcohol extract weighed 3·15 g. and was filtered off and washed with ether. The mother liquor (50 cc.) on evaporation gave a sticky residue mostly soluble in hot absolute alcohol and containing but a few centigrams of tryptophan.

The main portion of tryptophan (3·15 g.), corresponding to a yield of 1·7 per cent. on the caseinogen was remarkably pure, considering its simple method of separation. Apart from a little yellow pigment and a minute trace of tyrosine it seemed to be pure, as judged by its melting point, optical rotation and analysis.

A 1 per cent. solution, without needing clarification, showed a rotation of – 0·68° in a 2 dm. tube, hence $[\alpha]_D = -34°$. The recorded values vary from – 30 to – 40°, but the above figure agrees well with Hopkins and Cole's estimate of – 33°. On analysis (Kjeldahl) 13·9 per cent. nitrogen was found, compared with 13·72 per cent. calculated.

The preparation of tryptophan in a pure state in considerable quantities is an operation requiring some skill and experience in order to avoid loss of much of the product in mother liquors which crystallise with some difficulty. The extraction method it is believed will simplify the process to some extent and probably requires less careful manipulation. The crude product is already fairly pure and a single recrystallisation from dilute alcohol, as recommended by Hopkins, furnishes a completely pure substance. The method could probably be operated on a considerable scale and may be of service in obtaining tryptophan for bacteriological and other purposes.

II. A NEW DIBASIC AMINO-ACID OCCURRING IN PROTEINS.

It was found that after the separation of glutamic and aspartic acid as described above, large amounts of at least one other dicarboxylic acid were still present and could be isolated.

For simplicity in presentation, it is convenient to anticipate the experimental portion of the paper and to proceed at once to the main properties and probable constitution of the new acid.

(1) The acid is optically active and is extremely readily soluble in water, crystallising slowly in thick prisms from its syrupy solution. Most of its metallic salts, with the exception of those of silver and mercury, are also very soluble in water and do not crystallise readily but may be precipitated by alcohol. It forms a diethyl ester which has not yet been satisfactorily distilled without decomposition.

(2) The acid itself appears to possess the formula $C_5H_9O_5N$ and the salts of the heavy metals contain two equivalents of bases, while salts of the alkaline earths contain one.

(3) The acid behaves as a strong monobasic acid on titration with sodium hydroxide, using phenolphthalein as indicator and the results are in accord with a molecular weight of 163. The same molecular weight follows from the analysis of the well characterised silver salt, $C_5H_7O_5NAg_2$. In the presence of

excess of formaldehyde, the substance behaves towards sodium hydroxide as a dibasic acid.

(4) The acid, if not heated too strongly, contains the whole of its nitrogen in the (NH_2) form, as judged by its reaction towards nitrous acid. On prolonged heating over phosphorus pentoxide at 100–110°, a molecule of water is slowly eliminated and the nitrogen is converted into the (NH) form. This reaction is clearly analogous to the formation of pyrollidonecarboxylic acid from glutamic acid and is evidence of one carboxyl group being in the γ position relative to the amino-group.

(5) The acid or its salts on ignition give a definite but faint pyrrole reaction, especially if much charring occurs, thus favouring reduction. On the other hand, on heating with zinc dust, an intense pyrrole reaction is immediately obtained.

(6) On reduction with fuming hydriodic acid at 150°, glutamic acid and other products are obtained.

(7) The acid gives characteristic colour reactions with various phenols and concentrated sulphuric acid resembling aliphatic hydroxy-acids.

(8) On oxidation of the sodium salt of the acid (1 mol.) with chloramine-T (1 mol.) an aldehyde is obtained, presumably $C_4H_6O_4$, which on treatment with *p*-nitrophenylhydrazine gives a characteristic *osazone* containing one carboxyl group, the analysis of which agrees well with the formula $C_3H_3COOH(:N.NH.C_6H_4NO_2)_2$. The formation of the osazone from the aldehyde-acid is indicative of a hydroxyl group adjacent to the aldehyde group and hence contiguous to the amino-group in the original acid[1].

(9) No indication of lactone formation was observed, thus confirming the assumed position of the hydroxyl group as noted under (8) and excluding a γ position.

(10) On treatment with potassium cyanate a uramido-acid is formed which is not extractable from aqueous solution by ether. The uramido-acid, on heating with acids, gives an extremely soluble hydantoin, which is extractable by ether, thus resembling similar derivatives of aspartic and glutamic acid.

(11) The acid gives derivatives with phenyl isocyanate and β-naphthalenesulphonic chloride, but they are not well adapted for purposes of identification.

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[1] On oxidising the sodium salt with two molecules of chloramine-T, under the conditions which the writer used to convert glutamic acid into β-cyanopropionic acid [1917], a substance was obtained which on hydrolysis apparently furnished *malic acid*. The latter acid has only been identified thus far as calcium salt and by colour reactions. The reaction will be studied further.

The above results can hardly be reconciled with any other structure for the acid than that of an *α*-amino-*β*-hydroxyglutaric acid, *i.e.* *β*-hydroxyglutamic acid, $COOH\,.\,CH(NH_2)\,.\,CH(OH)\,.\,CH_2\,.\,COOH$.

An acid of the above structure containing two dissimilar asymmetric carbon atoms can exist in a variety of forms and it is not at all improbable that the substance isolated by me consists of more than one individual. It will be a matter of some difficulty to determine this as the melting point of the acid appears not to be sharp, partly owing to its rapid conversion into hydroxypyrrolidonecarboxylic acid, and its satisfactory recrystallisation has practical difficulties. Recently, however, a series of beautifully crystalline salts of the acid with strychnine, brucine and other alkaloids have been obtained and a further study of these products may prove of service. The formation of an unsaturated acid, $COOH\,.\,CH(NH_2)\,.\,CH = CH\,.\,COOH$, under the influence of alkaline or acid reagents, is obviously possible and such a product can be reconverted into the *β*-hydroxy-acid with partial loss of asymmetry. A study of the configuration and synthesis of the new acid and some of its biochemical relationships will be continued.

The amount of the new acid in caseinogen is very considerable, for no less than 10·5 g. of an almost wholly crystalline mass has been obtained from 100 g. of caseinogen after hydrolysis by sulphuric acid[1]. Hydrolysis of caseinogen by barium hydroxide in an autoclave at 140°, gave only about 1·5 per cent. of a syrup crystallising with difficulty but apparently containing some of the acid.

An acid of the structure indicated above has a number of interesting biochemical relationships which invite speculation. The formation from it by oxidation *in vivo* of malic and perhaps tartaric acid is clearly possible. Its relationship with serine is obvious and if it should be shown that the new acid may be formed *in vivo* by the oxidation of glutamic acid, an interesting parallelism with the known *β*-oxidation of fatty acids would be established. The writer [1913] has already drawn attention to the probable similarity in the behaviour in the animal body of glutamic acid and proline and the existence of *β*- or *γ*-hydroxyproline and *β*-hydroxyglutamic acid as protein constituents may well give a clue to the intermediary metabolism of the former. It is interesting in this connection to recall the old observation of Buisine showing that malic acid comprises 2·5 per cent. of the water-soluble matter of sheep sweat. It appears probable that this may originate from β-hydroxyglutamic acid. The biochemical interconversion of malic acid, lactic acid and glucose is well established. The easy formation of cyclic hydroxypyrrolidonecarboxylic acid from β-hydroxyglutamic acid may also be significant in connection with the synthesis of the pyrrolidine acids *in vivo.* But further knowledge of the new acid is clearly more desirable than premature speculation.

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[1] Osborne has drawn attention to the possible occurrence of hitherto unknown dicarboxylic acids in gliadin and glutenin owing to their disproportionately high yield of ammonia on hydrolysis, compared with their yield of aspartic and glutamic acids. These proteins will be examined for the new acid.

The writer believes that the substance under discussion is probably the first example of a hydroxy-dicarboxylic amino-acid occurring among the proteins. Reference must, however, be made to Skraup's statement as to the occurrence of hydroxyaspartic acid in caseinogen. As Fischer has pointed out, Skraup's experiments are not described in a form capable of repetition and no evidence, other than the analysis of a copper salt, is adduced that the minute amount of substance obtained by Skraup was a dicarboxylic acid. The value of this analysis for determining the basicity of the acid is small, since no carbon and hydrogen determinations were made on the free acid. If hydroxyaspartic acid, as described by Skraup, were obtainable from caseinogen, it would almost surely have accompanied the β-hydroxyglutamic acid, but no such acid was detected. Moreover, a search for tartaric or racemic acids among the products of the action of nitrous acid upon the dicarboxylic acids of caseinogen gave negative results, thus indicating the probable absence of hydroxyaspartic acid.

On the other hand, certain experiments carried out in Hopkins' laboratory, at Cambridge, by F. W. Foreman, read in the light of later work clearly indicate that a partial separation of the new acid had been accomplished. Foreman made use of the insolubility of the calcium salts of dibasic amino-acids in alcohol for their separation—a method which the writer has since utilised in a modified form. He obtained from the calcium salts crystalline aspartic and glutamic acid and a syrup soluble in acetic acid but difficult to crystallise, which contained pyrrolidonecarboxylic acid and "other unidentified substances probably of great importance." While the writer's experiments originated from a totally different direction and were not influenced by Foreman's observations, there is little doubt that if the latter had been able to pursue his investigations uninterruptedly, he would have isolated β-hydroxyglutamic acid.

EXPERIMENTAL.

Preparation of β-hydroxyglutamic acid. Four separate preparations of the acid have been made by different methods, and in each case a substance with similar properties was obtained. It is not proposed to describe the earlier less satisfactory methods, but simply to give an account of the method which hitherto has given the best result.

The material used was hydrolysed caseinogen thoroughly extracted with butyl alcohol in order to remove the monamino-monocarboxylic acids as described in the preceding section. Glutamic acid was next removed as completely as possible as hydrochloride by saturation with hydrogen chloride, taking care to resaturate the concentrated filtrate from the first crop of crystals and allowing the fluid to stand in an ice box for three days. The filtrate from the glutamic acid hydrochloride was then concentrated under reduced pressure, to remove as much hydrochloric acid as possible. The residual syrup was dissolved in water, digested with an excess of calcium hydroxide, filtered, concentrated to a syrup and then the calcium salts of the remaining dibasic acids were precipitated by alcohol. The calcium salts were then dissolved in water, the calcium removed with oxalic acid and the filtrate boiled with lead hydroxide to remove aspartic acid. The conduct of the preceding operations will be familiar to anyone accustomed to working with the amino-acids and are given in greater detail in the preceding section.

The filtrate from the lead aspartate (from 150 g. caseinogen) was freed from lead with hydrogen sulphide, diluted to 500 cc., acidified with 20 cc. of sulphuric acid and carefully precipitated with phosphotungstic acid to remove bases which are invariably present. The filtrate was then freed from sulphuric and phosphotungstic acids with barium hydroxide in the usual way and the excess of the latter removed fairly exactly with sulphuric acid. The volume of the filtrate, which was strongly acid, was brought to about half a litre. In the earlier experiments the new acid was precipitated with mercuric acetate and sodium carbonate, but later it was found that the silver salt was preferable.

Small amounts of hydrochloric acid are invariably present in the solution and this was first removed by adding dilute nitric acid (5 cc.) and then silver nitrate until no more precipitation occurred. The filtrate from the silver chloride was then made neutral or feebly alkaline with sodium hydroxide. The new acid was then precipitated by the alternate addition of strong silver nitrate solution (15 per cent.) and approximately normal sodium hydroxide. A heavy white precipitate was soon produced in the solution and more silver nitrate or sodium hydroxide was added as long as addition of either solution produced a white precipitate. The sodium hydroxide is best added by degrees from a burette and a large excess must be avoided since it decomposes the precipitate. When excess of silver nitrate was present and precipitation completed, a test portion of the filtrate, on adding a drop of sodium hydroxide solution, gave only brown silver oxide. The end point is easily determined with a little care. The precipitated silver salt, which at first was pure white but on completion of the operation was light brown, from a little silver oxide, was filtered off using suction and washed with cold water. It was then decomposed with hydrogen sulphide and the filtrate concentrated under reduced pressure. It is probably advisable to avoid heating the solution unduly in order to limit any formation of hydroxypyrrolidonecarboxylic acid. A clear yellowish syrup containing the acid was obtained and the separation of crystals only commenced after most of the water had been removed in the vacuum desiccator. Gradually thick prisms begin to separate and eventually the whole is transformed into a solid mass of crystals. The crystalline magma from 150 g. of caseinogen weighed 19·5 g. and contained, as judged by titration with standard alkali and phenolphthalein, 17·5 g. of the acid. The acid is difficult to crystallise so that a convenient amount of mother liquor may be removed and a fairly good plan is to crush the crystals separated from water with a mixture of one part of glacial acetic acid and four parts methyl alcohol and finally to wash with methyl alcohol. Precipitation of the aqueous solution with alcohol gives a syrup which rapidly solidifies, and the same is true on adding alcohol to the concentrated acetic acid solution. The acid is extremely soluble in water, dissolving in about its own weight of water, and is readily soluble in acetic acid, slightly soluble in methyl alcohol but practically insoluble in ethyl alcohol, ether, or ethyl acetate. In aqueous solution the substance shows a low dextro-rotation which is increased on addition of hydrochloric acid. Its optical properties will be examined more carefully and reported later, as variation in concentration appears to influence the specific rotation considerably.

For analysis, the acid was dried in vacuo over phosphorus pentoxide at a temperature not above 40°:

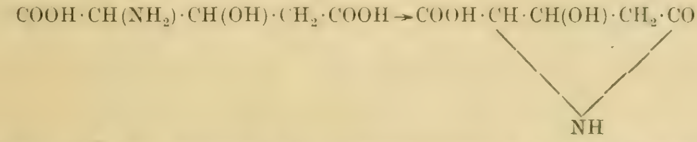
0·1298 g.; 0·1741 g. CO_2, 0·0661 g. H_2O
0·2314 g. neutralised 13·9 cc. $N/10$ NaOH (Kjeldahl)
0·0195 g. gave 3·00 cc. N at 23° and 770 mm. (van Slyke)

	C	H	N	
Found ...	36·6	5·66	8·40	8·45 (van Slyke)
Calculated for $C_5H_9O_5N$	36·8	5·52	8·59	

Other analyses on less pure material gave results varying from 35·8–37·7 per cent. C, 5·6–6·2 per cent. H, 8·4–9·3 per cent. N.

Molecular Weight. 0·2071 g. of the acid dissolved in water and titrated with *N*/10 NaOH required 11·6 cc. when sensitive litmus paper was used as indicator and 12·3 cc. with phenolphthalein. Assuming that the acid reacts like glutamic acid as a monobasic acid, these figures correspond to molecular weights of 171 and 160 respectively, with a mean of 166. Another specimen of the acid (0·240 g.) required 14·2 cc. of alkali using litmus as indicator, equivalent to a molecular weight of 168. Analysis of the silver salt (see later) gave 57·3 per cent. Ag corresponding to a molecular weight of 163. The calculated value for $C_5H_9O_5N$ is 163.

The behaviour of the acid on heating is peculiar. In the neighbourhood of 100° the acid becomes pasty and appears to part with water. Eventually on raising the temperature the whole of the acid is converted into a clear glassy mass. The change is brought about rapidly at a temperature of about 140–150°, but can be effected in a few hours by heating over phosphorus pentoxide at 110°. Most of the nitrogen in the glassy compound, as already stated, is no longer in the (NH_2) form and no longer reacts with formaldehyde or nitrous acid. A sample of the product heated for 6 hours at 110° gave the following result on analysis: C 39·5 per cent., H 5·7 per cent., total N 9·4 per cent., amino-N 4·3 per cent. The results are obviously explainable on the basis of a mixture of β-hydroxyglutamic acid and hydroxypyrrolidone-carboxylic acid. The latter acid contains 41·3 per cent. C, 4·9 per cent. H, 9·86 per cent. total N and no (NH_2) nitrogen.



$$\mathrm{COOH\cdot CH(NH_2)\cdot CH(OH)\cdot CH_2\cdot COOH \rightarrow COOH\cdot \underbrace{CH\cdot CH(OH)\cdot CH_2\cdot CO}_{NH}}$$

Silver Salt. This salt was used for the separation of the acid. A specimen for analysis was prepared, as already described, by adding silver nitrate (2 mols.) to the sodium salt of the acid (1 mol.) in about 5 per cent. solution. It separated as a granular very sparingly soluble pure white heavy precipitate showing crystalline structure on standing. It was filtered off and washed with water, alcohol and ether and dried *in vacuo* in the dark. The salt is not excessively sensitive to light. It was analysed with the following result:

0·2568 g.; 0·1495 g. CO_2; 0·0440 g. H_2O

0·1415 g. neutralised 3·6 cc. *N*/10 acid (Kjeldahl)

0·2976 g.; 0·1707 g. Ag.

	C	H	N	Ag.
Found	15·8	1·90	3·56	57·3
Calculated for $C_5H_7O_5NAg_2$	15·9	1·85	3·70	57·3

Copper Salt. The acid forms an extremely soluble copper salt on boiling with precipitated copper oxide and this fact was utilised in preliminary experiments, in order to be assured of the absence of aspartic, glutamic and other acids which form sparingly soluble copper salts. None of these latter salts was ever detected as an impurity in the new acid, and on the whole, the copper salt, for purposes of purification, is much less satisfactory than the silver salt. To prepare the copper salt, the acid was boiled in 2 per cent. solution with a large excess of well washed copper oxide for an hour under a reflux condenser. On filtering and concentrating, the syrup slowly solidified, giving some crystalline product which was hard to separate. It was found more convenient to add methyl alcohol by degrees to a fairly strong solution of the salt, stirring during the addition. The bulk of the copper salt is precipitated as a clear blue powder and a few crystals are apt to separate from the mother liquor on standing. A more complete precipitation is effected by ethyl alcohol, but methyl alcohol is preferred as the copper salt is slightly soluble in it if a little water is present and possibly a certain amount of purification may be so effected. The salt was dried at 110° and contained only traces of water. It was analysed with the following result:

0·1759 g.; 0·1681 g. CO_2; 0·0533 g. H_2O
0·1093 g.; 0·0379 g. CuO
0·1265 g. neutralised 5·8 cc. *N*/10 acid (Kjeldahl)
0·0120 g.; 1·31 cc. N. at 23° and 764 mm. (van Slyke).

	C	H	N	Cu
Found	26·1	3·36	6·42; 6·15	27·7
Calculated for $C_5H_7O_5NCu$	26·7	3·12	6·23	28·3

Mercury Salts. Mercuric chloride added to a solution of the acid produces no precipitate, but on adding excess of sodium acetate, a microcrystalline salt soluble in hot water is precipitated. It has not yet been analysed. The alternate addition of mercuric acetate and sodium carbonate, as practised by Neuberg and Kerb, precipitates the acid practically quantitatively, as a white amorphous salt of complex composition, and use has been made of this property for the separation of the acid. Mercurous salts also precipitate neutral solutions of the acid.

Lead Salt. The lead salt was prepared by boiling the acid in 2 per cent. aqueous solution for one hour with excess of well washed freshly precipitated lead hydroxide. The filtered and concentrated solution did not readily crystallise but dried up to a heavy clear glassy crystalline mass easily soluble in water but insoluble in alcohol. A sample, dissolved in water and precipitated with alcohol, was obtained as a heavy white granular powder.

0·1350 g.; 0·0824 g. PbO.

Found ... ... 56·6 per cent. Pb

Calculated for $C_5H_7O_5N$ Pb 56·3 ,,

Cadmium and Zinc Salts. These salts were prepared in the same fashion as the lead salt. Neither of them crystallises readily and they are extremely soluble in water but insoluble in alcohol. They appear to be normal salts of the type $C_5H_7O_5N\ M''$.

Calcium Salt. The free acid was warmed with excess of lime water and filtered. On concentrating on the water bath calcium carbonate and hydroxide separated out and the operation of filtration and evaporation was twice repeated. It appears that a normal calcium salt is first formed which decomposes on exposure to air to give the acid salt. The concentrated solution of the calcium salt refused to crystallise, and it was therefore precipitated with alcohol. The soft amorphous precipitate soon became hard and on drying *in vacuo* at 110° the substance became crystalline resembling fused sodium acetate. It is extremely soluble in water, insoluble in alcohol.

0·1098 g.; 0·0428 g. $CaSO_4$.

Found ... ... 11·5 per cent. Ca

Calculated for $(C_5H_8O_5N)_2Ca$ 11·0 ,,

Barium Salt. The acid barium salt was obtained by boiling the acid with excess of precipitated barium carbonate. The filtered solution on concentrating dries to a hard gritty mass. The salt is easily soluble in water, insoluble in alcohol. It was dried at 110°.

0·2241 g.; 0·1112 g. $BaSO_4$.

Found ... ... 29·2 per cent. Ba.

Calculated for $(C_5H_8NO_5)_2Ba$ 29·7 ,,

β-Naphthalenesulphonic derivative. One gram of the acid was dissolved in 6 cc. of normal sodium hydroxide and shaken with β-naphthalenesulphonic chloride (2·7 g.) dissolved in ether (15 cc.). Additional sodium hydroxide (30 cc.) was added by degrees with vigorous shaking. The mixture was allowed to stand overnight, and the aqueous portion filtered and precipitated with concentrated hydrochloric acid (6 cc.). A clear oil separated which became sticky on washing with water, and on long standing eventually solidified to a hard crystalline mass. It is sparingly soluble in water but readily soluble in alcohol and acetone, but all attempts to crystallise it satisfactorily failed. It was purified to some extent by redissolving in *N* NaOH and again precipitating with acid. The substance was found to contain 4·04 per cent. of nitrogen (Kjeldahl) compared with a calculated value of 3·96.

On dissolving the naphthalenesulphonic derivative in a minimum (1 mol.) of warm $N/10$ NaOH and adding copper acetate, a curdy light green copper salt, very sparingly soluble in water, was at once precipitated. It was dried at 110° and analysed as follows:

0·1036 g.; 0·0201 g. CuO
0·1027 g.; 0·0193 g. CuO
0·2098 g. neutralised 5·0 cc. $N/10$ acid (Kjeldahl).

	Cu	N
Found	15·4, 14·9	3·34
Calculated for $C_{15}H_{13}O_7NSCu$	15·3	3·37

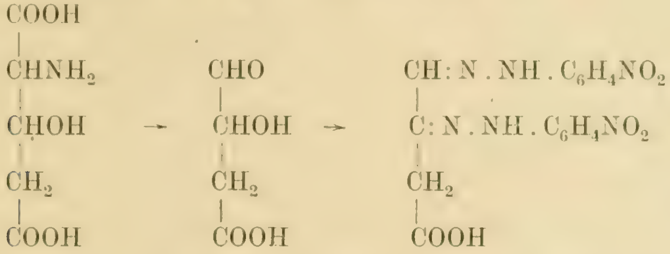
Oxidation of the acid with Chloramine-T. A normal solution (5 cc.) of the acid, containing 0·815 g., was neutralised with normal sodium hydroxide (5 cc.) and then mixed with 1·44 g. chloramine-T (sodio-*p*-toluenesulphochloroamide), dissolved in 5 cc. of water. An immediate reaction took place with precipitation of toluenesulphonamide, but active chlorine did not disappear until after about an hour. The solution was filtered and then warmed on the water-bath with a solution of *p*-nitrophenylhydrazine (2 g.) in dilute alcoholic sulphuric acid. On heating, it appeared that the hydrazone first formed was being converted into a sparingly soluble osazone. After three-quarters of an hour, the precipitate was filtered off with suction and well washed with alcohol. The washed product, weighing about 0·6 g., was recrystallised from boiling nitrobenzene, and separated out as red-brown prismatic needles melting at 297–299°. The substance is sparingly soluble in most neutral solvents but is very freely soluble in pyridine giving a yellow crystalline salt. Alcoholic ammonia gives a red crystalline salt. On adding sodium hydroxide to an alcoholic solution containing a trace of the substance, a deep blue colour is produced. This reaction is apparently characteristic of two adjacent nitrophenylhydrazine groups.

For analysis, the substance was dried *in vacuo* at 120° over phosphorus pentoxide.

0·1142 g.; 0·2140 g. CO_2; 0·0397 g. H_2O
0·1250 g.; 0·2353 g. CO_2; 0·0432 g. H_2O
0·1650 g.; 34·0 cc. N at 26° and 756 mm. (Dumas).

	C	H	N
Found	51·1, 51·3	3·86, 3·84	22·7
Calculated for $C_{16}H_{14}O_6N_6$	51·1	3·72	22·6

Assuming the acid to be correctly represented as a β-hydroxyglutamic acid, the course of the reaction with chloramine-T[1] and the subsequent osazone formation would almost surely be represented as follows:



$$\begin{array}{lclcl} \mathrm{COOH} & & & & \\ | & & & & \\ \mathrm{CHNH_2} & & \mathrm{CHO} & & \mathrm{CH:N.NH.C_6H_4NO_2} \\ | & & | & & | \\ \mathrm{CHOH} & \rightarrow & \mathrm{CHOH} & \rightarrow & \mathrm{C:N.NH.C_6H_4NO_2} \\ | & & | & & | \\ \mathrm{CH_2} & & \mathrm{CH_2} & & \mathrm{CH_2} \\ | & & | & & | \\ \mathrm{COOH} & & \mathrm{COOH} & & \mathrm{COOH} \end{array}$$

No attempt to isolate the free aldehyde has yet been made, but this will be tried later, as well as its conversion into malic acid.

Reduction of the Acid with Hydriodic Acid. The reduction of hydroxyglutamic acid with hydriodic acid presents some difficulties. In all the experiments, one part of the acid (0·5–1 g.) was heated with 0·3 part red phosphorus and 10 parts of fuming hydriodic acid (sp. gr. 1·9). At a low temperature, such as 120° on boiling under a reflux condenser, little or no change occurs, whilst in a sealed tube at high temperatures (180–200°) any glutamic acid formed is decomposed with formation of butyric and other products [cf. Kwisda, 1891]. On the other hand for the reduction of β-hydroxyglutaric acid by hydriodic acid, a temperature of 180° is desirable, according to Pechmann and Jenisch [1891]. The best result was obtained by heating the mixture in a sealed tube at 145–150° for six hours. The clear contents of the tube were diluted with water (100 cc.) and boiled with excess of precipitated lead hydroxide until neutral. After filtering, the lead was removed from the filtrate with hydrogen sulphide and the solution concentrated to a syrup. Fuming hydrochloric was then added and the mixture allowed to stand in the ice box. Characteristic crystals of glutamic acid hydrochloride soon separated and they were filtered off and recrystallised again from hydrochloric acid. The crystals melted at 195–197° and a mixed melting point determination with *d*-glutamic acid hydrochloride of other origin melted at the same temperature. The yield of pure product in two experiments was only between 10 and 15 per cent. The substance gave the pyrrole reaction strongly. It was analysed for nitrogen by van Slyke's method:

0·0132 g.; 1·75 cc. N at 21° and 765 mm. = 7·57 per cent. N.
Calculated for $C_5H_9O_4N$ HCl = 7·65 per cent. N.

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[1] For analogous oxidations of aspartic and glutamic acids with chloramine-T see Dakin [1917].

Colour Reactions of the Acid. On adding excess of concentrated sulphuric acid to a few drops of a dilute solution of the acid together with a few milligrams of a phenol, a variety of rather definite colour reactions are obtained some of which may be of use for purposes of identification. Perhaps the reactions with resorcinol and *α*-naphthol are the most striking and sensitive. It should be noted that the absence of any trace of nitrates is essential or misleading results will be obtained.

Resorcinol. Clear reddish purple turning brown on warming.

Catechol. Blue green.

Thymol. Clear bright green.

Pyrogallol. Dark green.

Phloroglucinol. Cherry red.

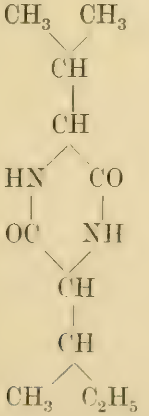
α-Naphthol. Clear fluorescent green.

β-Naphthol. On warming, a yellowish green fluorescent solution resembling the similar reaction with malic acid.

The acid also appears to give a red colour reaction with diazobenzenesulphonic acid when warmed with excess of sodium hydroxide. This reaction due to Rosenthaler [1912] is given by various alcohols and especially by malic acid. Minute traces of histidine are apt to complicate the reaction.

III. A NEW PEPTIDE, ISOLEUCYLVALINE ANHYDRIDE, OBTAINED BY THE ACID HYDROLYSIS OF CASEINOGEN.

In the first of the preceding sections, an account was given of the separation of certain peptide anhydrides from the crude butyl alcohol extract from hydrolysed caseinogen. The peptide mixture is fairly easily soluble in butyl alcohol and was separated from proline by successive crystallisation from absolute alcohol and water—the proline remaining in solution. The two fractions, from alcohol and water respectively, were mixed together and averaged about 5 per cent. of the weight of caseinogen originally hydrolysed. The mixture is evidently composed of a number of different compounds and would repay careful examination. A tyrosine-containing peptide was separated by the action of cold normal sodium hydroxide and subsequent precipitation with acetic acid. Its complete identification still remains to be accomplished. The substance remaining insoluble in cold dilute alkali was recrystallised first of all from boiling absolute alcohol, in which it is sparingly soluble, and subsequently from 50 per cent. acetic acid, in which it dissolves readily. The crystalline product thus obtained was in the form of woolly masses of fine needles, often over 1 cm. in length, and appeared perfectly uniform under the microscope. The yield was considerable, amounting in one case to slightly over 1 per cent. of pure product, so that the actual amount present must have been considerably more. The substance was almost insoluble in water and in dilute acid or alkali, even on moderate heating. In cold 50 per cent. acetic acid, its solubility was very close to 0·35 per cent., but much greater in the hot acid. It was sparingly soluble in alcohol, acetone or ethyl acetate, and insoluble in ether. It dissolved in concentrated sulphuric acid without developing colour. The substance did not react when boiled with copper oxide to give a copper salt, nor did it evolve nitrogen readily when treated with nitrous acid. These reactions clearly indicated the presence of the diketopiperazine type of linkage. At first the substance was thought to be leucinimide, but this was excluded by both melting point and analysis. The results of the analysis agreed with a *l*-leucyl-*d*-valine anhydride and such a substance has been isolated by Abderhalden and Funk [1907] from caseinogen and has been synthesised by Fischer and Scheibler [1908]. The melting points given for these products agree closely with one another, namely 285° (uncorr.) and 282° (corr.) respectively. On the other hand, the melting point of the substance under investigation was constant at 310–312° (uncorr.), so that there could be no question of identity, though possibly of stereoisomerism. But on hydrolysing the new substance with hydrobromic acid, good evidence was obtained of the presence of both *d*-isoleucine and *d*-valine, so that the original peptide was undoubtedly a *d*-isoleucyl-*d*-valine anhydride, *i.e.* isopropyl-*sec*-butyldiketopiperazine with the following structure:



Analysis. The substance was recrystallised from 50 per cent. acetic acid dried *in vacuo* at 110°.

0·1003 g.; 0·2281 g. CO_2; 0·0884 g. H_2O
0·1030 g.; 0·2343 g. CO_2; 0·0858 g. H_2O
0·1000 g. neutralised 9·45 cc. $N/10$ acid (Kjeldahl).

	C	H	N
Found	62·2, 62·0	9·79, 9·26	13·2
Calculated for $C_{11}H_{20}O_2N_2$	62·2	9·5	13·2

Optical Rotation. A 1 per cent. solution of the substance in glacial acetic acid was examined. Two different specimens gave the following values:

$$c = 1·0,\ l = 2 \text{ dm.},\ \alpha = -0·87° \text{ hence } [\alpha]_D^{20°} = -43·5°,$$

$$c = 1·0,\ l = 2 \text{ dm.},\ \alpha = -0·84° \quad ,, \quad [\alpha]_D^{20°} = -42·0°.$$

Hydrolysis. One gram of the peptide was heated in a sealed tube with 10 cc. hydrobromic acid (sp. gr. 1·48) at 115–120° for eight hours. A little brown insoluble matter was filtered off and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in water and made just alkaline to ammonia and allowed to crystallise. The crystalline residue was washed with a little water and alcohol and dried at 90°. The mixed amino-acids were analysed for nitrogen and examined optically.

0·1351 g. neutralised 10·7 cc. $N/10$ acid (Kjeldahl) = 11·09 per cent. N.

0·1502 g. dissolved in 10 cc. of 20 per cent. hydrochloric acid, showed a dextro-rotation of + 0·96° in a 2 dm. tube, hence $[\alpha]_D = +32·1°$.

It will be seen that these figures agree closely with the mean of the values for *d*-valine and *d*-isoleucine. *d*-Valine contains 11·97 per cent. nitrogen and has a rotation of + 29·6°, while *d*-isoleucine contains 10·69 per cent. of nitrogen and has a rotation in hydrochloric acid of + 36·8°. The fact that the rotation of the mixed amino-acids from the peptide had as high a rotation as + 32·1°, is almost conclusive evidence of the presence of *d*-isoleucine and *d*-valine, as will be readily seen on consulting a table of optical rotation constants for the amino-acids.

The separation of *d*-isoleucine and *d*-valine is a difficult matter, especially when only small amounts of material are available. A partial separation of the residual amino-acid mixture was accomplished by taking out a fraction containing most of the *d*-isoleucine as a lead salt, according to Levene and van Slyke's method. The filtrate containing most of the valine was treated with hydrogen sulphide and recovered as described by the above authors. Since the optical properties of the two fractions were of greater importance than analytical figures, the rotations were observed first and after reading nitrogen estimations made on aliquot parts of the solution.

The crude *d*-isoleucine, 0·1200 g., dissolved in 10 cc. of 20 per cent. hydrochloric acid, showed a rotation of + 0·82° in a 2 dm. tube, hence $[\alpha]_D = +34{\cdot}2°$. It contained 10·90 per cent. of nitrogen. The crude *d*-valine contained 11·31 per cent. of nitrogen and 0·1500 g. in 10 cc. of 20 per cent. hydrochloric acid showed a dextro-rotation of + 0·92°, hence $[\alpha]_D = +30{\cdot}7°$. While the comparison of the above figures with the calculated shows that the separation of the isoleucine and valine was incomplete, it also appears to offer satisfactory evidence of the presence of both of them in about equal amounts.

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XXVII. OBSERVATIONS ON SOME METALLIC COMPOUNDS OF HAEMATOPORPHYRIN.

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INTRODUCTION.

It has long been recognised that haematoporphyrin forms at least three distinct types of compounds. It unites with mineral acids to form crystalline salts, *e.g.* the hydrochloride [Zaleski, 1902], in which the pigment plays the part of a base or cation, secondly it unites with strong bases to form salts, *e.g.* sodium, potassium and ammonium compounds [Nencki and Sieber, 1888], in which the pigment functions as an acid or anion, and thirdly it forms so-called metallic compounds, in which the metals enter into a more or less stable organic combination with the pigment, the metals ceasing to exist as ions. The absorption spectrum of haematoporphyrin in alkaline solution is probably to be regarded as that of the anion and the spectrum of the pigment in acid solution as that of the cation. An absorption spectrum of neutral haematoporphyrin has also been described, which may be that of the non-ionised pigment [Garrod, 1896]. The first two types are readily convertible into one another, while the pigments belonging to the metallic type are comparatively stable substances, their formation being in many cases an irreversible reaction.

The present paper deals mainly with the third class of compounds, being, in the first instance, an investigation of the spectroscopic characters of a number of synthetic compounds of pure haematoporphyrin, prepared by Nencki's method [Nencki and Zaleski, 1900], with various metallic hydroxides. The work of previous observers will be referred to in connection with each pigment.

A diffraction spectroscope was chosen for making the observations, since it yields a normal spectrum and the measurement of wave lengths is therefore greatly facilitated. On the other hand, there are certain disadvantages in the use of a diffraction spectroscope as compared with a prismatic one for the observation of absorption spectra. The relatively great dispersion in the red region of the diffraction, as compared with the prismatic, spectrum results in absorption bands in that area being spread over a greater length with a consequent reduction in their intensity. The fact that the sensitivity of the eye for red rays is very low, the region of maximal sensitivity being about 530$\mu\mu$ [Ebert, 1888], further increases the difficulty of visual observation in this region. The question of the comparative efficiency of prismatic and diffraction spectra is fully discussed by Kayser [1900], from whom the following quotation is taken.

"Let us consider a prismatic spectrum, which has the same dispersion in the middle of the visible region as a diffraction spectrum, then it will be found that the prismatic spectrum while relatively extended in the blue and violet region is contracted in the red. Since it is just the latter parts which have a low intensity of light, observation in the prismatic spectrum is still possible, when it has ceased to be so in the diffraction spectrum."

Prism spectroscopes are therefore on the whole better adapted for the visual examination of pigments in gradually decreasing molecular concentrations, especially when the pigments show absorption bands in the red region. My original intention was to prepare "absorption curves" or "curves of molecular vibrations" according to Hartley's method [Kayser, 1905; Baly, 1905, p. 410] with the aid of a prism spectroscope, but delay in obtaining suitable apparatus has compelled me to limit the present observations to solutions of only one or two molar concentrations. Although the observations are therefore incomplete, yet they have the advantage over previous analogous investigations of allowing one to compare similar molecular concentrations of the pigments. I hope shortly to be in a position to supplement the following results by plotting out the absorption curves of the pigments with the aid of a prism spectroscope provided with means for the rapid and accurate determination of wave lengths.

The instrument used for the following work was a spectrometer provided with a graduated circle reading by opposite verniers to one minute of arc, and a transparent diffraction grating containing 14,500 lines to the inch.

The source of light employed was a small carbon arc lamp, the light being focussed on the slit of the spectroscope. The plane of the grating was adjusted so as to be perpendicular to the incident light.

The wave lengths (λ) of the margins of the absorption bands are stated in $\mu\mu$ or 10^{-7} cm.

Two stock solutions of haematoporphyrin of $M/1000$ concentration were prepared, one an acid and the other an alkaline solution. In calculating the molar concentrations Willstätter's [Willstätter and Stoll, 1913, pp. 44, 406] formula, $C_{33}H_{38}N_4O_6$; M.W. = 586·349, was adopted as a basis.

0·0586 g. of pure haematoporphyrin was dissolved in glacial acetic acid, 1 g. of pure sodium acetate was added, and the solution was made up to 100 cc. with glacial acetic acid (Solution 1). An alkaline solution of the same concentration was made up by dissolving 0·0586 g. of haematoporphyrin in 85 per cent. alcohol containing 10 cc. of $N/10$ caustic potash, the solution being made up to 100 cc. in a standard flask with 85 per cent. alcohol (Solution 2).

The absorption spectra of haematoporphyrin in alkaline and in mineral acid solutions are first recorded for the sake of comparison with their metallic derivatives.

$M/10{,}000$ haematoporphyrin in alkaline solution prepared by diluting Solution 2 with 85 per cent. alcohol.

Thickness of layer	Band I	Band II	Band III	Band IV
25 mm.	624·5–615·4	575–561	540·3–527·8	508·9–491·4

10 cc. of Solution 1 were made up to 100 cc. with 85 per cent. alcohol containing sulphuric acid (2 cc. of the concentrated acid in 100 cc. alcohol). The resulting solution is therefore $M/10{,}000$, and shows the following absorption bands.

Thickness of layer	Band I	Band II
25 mm.	600·7–589·3	$\underbrace{577{\cdot}3\text{–}560{\cdot}5}_{\text{Faint absorption}}\underbrace{\text{–}540{\cdot}3}_{\text{Dark absorption}}$

I. *The conditions under which the metallic compounds are formed.*

Haematoporphyrin was found to combine with certain metals in the presence of excess of alkali or in acetic acid solution. When the metallic hydroxide was soluble in ammonia, that alkali was used. On the other hand, it is necessary to employ caustic soda or caustic potash as the alkali in the preparation of the lead compound. No combination takes place in the presence of free mineral acids. When salts of the metals with mineral acids are added to solutions of haematoporphyrin in glacial acetic acid, it is therefore necessary to add at least an equivalent amount of sodium acetate.

II. *Compounds of haematoporphyrin with certain metals.*

1. *Zinc and Cadmium compounds.*

The absorption spectrum of the zinc compound was first described by MacMunn [1889], later by Garrod [1892], and Hammarsten [1893]. It was prepared by the action of zinc chloride on an ammoniacal solution of haematoporphyrin. A $M/5000$ solution of the zinc compound in ammoniacal alcohol shows the following absorption bands.

Thickness of layer	Band I	Band II
25 mm.	591·2–569·2	559·1–527·3
10 mm.	587·4–572·1	551–534

The zinc compound can also be easily prepared as a $M/5000$ solution in glacial acetic acid in the following way. 0·01 g. of zinc acetate is added to 10 cc. of $M/1000$ solution of haematoporphyrin in glacial acetic acid. The solution is then heated for about 20 minutes on the water-bath, cooled, and made up to 50 cc. with glacial acetic acid.

The following was its absorption spectrum:

Thickness of layer	Band I	Band II
10 mm.	585–568	550–532

A $M/5000$ solution of the cadmium compound was prepared in the following way. 10 cc. of the $M/1000$ alkaline solution of haematoporphyrin were mixed with 5 cc. of concentrated ammonia, and 0·2 cc. of $M/1$ cadmium chloride, and the solution made up to 50 cc. with 50 per cent. alcohol. After standing at room temperature for 24 hours, the solution showed the following absorption bands on spectroscopic examination.

Thickness of layer	Band I	Band II
25 mm.	596·4–578·8	560·5–542·74
10 mm.	Too faint to define	556·2–543·2

The cadmium compound cannot be prepared in solution in glacial acetic acid. Both the zinc and cadmium compounds are unstable substances, being at once converted into acid haematoporphyrin on acidification with hydrochloric or sulphuric acid.

2. *Compounds of haematoporphyrin with nickel, cobalt and iron.*

These compounds are most readily prepared from solutions of haematoporphyrin in glacial acetic acid, but can also be prepared from ammoniacal solutions. The latter two compounds were first prepared by Laidlaw [1904], and the nickel compound by Zaleski [1904].

A $M/5000$ solution of the nickel compound was prepared in the following way. 5 cc. of a $M/1000$ solution of haematoporphyrin in glacial acetic acid were mixed with 5 cc. of N sodium acetate dissolved in glacial acetic acid and 0·005 g. of nickel acetate. The solution was then heated on the water-bath for about half-an-hour, allowed to cool and made up to 25 cc. with glacial acetic acid. The cobalt compound was prepared in a similar way, 0·01 g. of cobaltous chloride being added instead of the nickel salt.

These solutions showed the following absorption bands on spectroscopic examination.

M/5000 NICKEL COMPOUND.

Thickness of layer	Band I	Band II
10 mm.	558·15–543·7	(522·9–510)?

M/5000 COBALT COMPOUND.

25 mm.	567·8–552·9	539·4–515·2
10 mm.	564·9–553·3	(531·15–516·15)?

In both cases the first bands are well defined and persist on considerable dilution. The second bands are much fainter and their margins are difficult to define with the diffraction spectroscope. The absorption spectra of both pigments bear a striking resemblance to that of haemochromogen. The addition of mineral acid to solutions of these compounds does not produce any distinct change in their absorption spectra. They differ, therefore, from the zinc and cadmium compounds in being stable in the presence of the mineral acid. The following experiments prove this statement: 25 cc. of M/5000 solutions of the pigments were mixed with an equal volume of 85 per cent. alcohol strongly acidified with sulphuric acid (10 cc. of the concentrated acid made up to 100 cc. with 85 per cent. alcohol). The resulting solutions were therefore M/10,000.

NICKEL COMPOUND.

Thickness of layer	Band I	Band II
25 mm.	560·55–542·7	523·9–507·4?

COBALT COMPOUND.

25 mm.	567·3–553·3	533·6–519·2?

The synthetic compound of haematoporphyrin with iron is somewhat more difficult to prepare. The following method was found to lead to the desired result: 0·03 cc. of M/2 ferric chloride solution was placed in a 25 cc. flask, and 10 cc. of glacial acetic acid and a trace of pure iron wire were added to convert the ferric into a ferrous salt. The addition of metallic iron is essential. No interaction was found to take place between haematoporphyrin and iron when present as a ferric salt. On the other hand Willstätter and Stoll [1913, p. 412] have proved that iron in the ferric form can be linked with aetioporphyrin in the presence of an equivalent amount of sodium acetate. The solution was then heated to the boiling point on the oil-bath for some minutes. 5 cc. of *M*/1000 haematoporphyrin dissolved in glacial acetic acid were added and the fluid was maintained nearly at the boiling point for fully an hour. Finally the solution was cooled and made up to 25 cc. with glacial acetic acid. An absorption band in the red region of the spectrum closely resembling in position and character the corresponding band of acid haematin can easily be distinguished on examination with a direct vision spectroscope. This band is somewhat difficult to define on examination with the diffraction spectroscope.

M/5000 SYNTHETIC HAEMATIN IN ACID SOLUTION.

Thickness of layer	Band 1
25 mm.	633–611·69

A *M*/5000 solution of pure haematin (M.W. 621·14 Willstätter), prepared from crystalline haemin, in glacial acetic acid showed the following absorption band in the red region on examination with the diffraction spectroscope. A layer of 45 mm. was found to be necessary in order to define the limits of the band with the diffraction spectroscope.

Thickness of layer	Band I
45 mm.	647·2–612

Although the fact, that the thicknesses of the layers examined were different for the two pigments, renders comparison difficult, it will be noticed that the absorption band lies farther towards the extreme red end of the spectrum in the case of natural haematin than in that of the synthetic pigment.

Since the absorption bands of reduced haematin in alkaline solution are better defined and more persistent than those of acid haematin, it was considered necessary to compare the reduced forms of the synthetic and natural pigments in alkaline solution.

Synthetic reduced haematin or haemochromogen in alkaline solution was therefore prepared in the following way from haematoporphyrin. 5 cc. of *M*/1000 haematoporphyrin dissolved in glacial acetic acid, 0·03 cc. of *M*/2 solution of ferric chloride and a trace of iron wire were heated in the same way as the previous preparation. The solution was cooled and rendered alkaline by the very gradual addition of concentrated ammonia, the solution being cooled from time to time by immersion in cold water. 0·3 g. of hydrazine hydrochloride was then added and the solution was made up to 25 cc. with 50 per cent. ethyl alcohol. This *M*/5000 solution of reduced synthetic haematin in alkaline solution or haemochromogen showed the following absorption bands.

Thickness of layer	Band I	Band II
25 mm.	$\underbrace{562{\cdot}47\text{–}556{\cdot}2}_{\text{Faint absorption}}\underbrace{\text{–}539{\cdot}4}_{\text{Dark absorption}}$	526·8–506·9
10 mm.	552·9–543·2	524·4–512·8

Band II is much less intense and well defined than Band I.

For comparison with the synthetic pigment, a *M*/5000 solution of haemochromogen was prepared from pure haematin in the following way: 0·1553 g. of pure haematin (M.W. = 621·14) [Willstätter and Stoll, 1913, p. 35] was placed in a 100 cc. flask, dissolved in 70 per cent. ethyl alcohol containing 2 cc. of *N* caustic soda, and the solution was made up to 100 cc. with 70 per cent. alcohol. The concentration of this solution was therefore *M*/400. 4 cc. of this solution were placed in a 50 cc. flask, 5 cc. of *N* caustic potash and 0·5 g. of hydrazine hydrochloride were added. Finally the solution was made up to 50 cc. with *N* ammonia. This *M*/5000 solution of haemochromogen showed the following absorption bands on spectroscopic examination.

Thickness of layer	Band I	Band II
25 mm.	566·3–542·3	529·7–508·4
10 mm.	557·7–545·15	527·3–513·7

The absorption spectra of natural and synthetic haematin are therefore similar, but not identical.

The similarity of their absorption spectra indicates that the nickel and cobalt compounds are closely related to the reduced form of haematin. They differ from haemochromogen in their stability, being resistant to the action of mineral acids and unaffected by exposure to air. Haemochromogen, on the other hand, is rapidly oxidised to haematin on exposure to air. The stability of the nickel and cobalt compounds is probably due to the fact that both metals form stable divalent compounds; while divalent iron tends to undergo conversion into the trivalent form. Haemochromogen prepared from haematin is so unstable in acid solution, that special precautions have to be taken in its preparation [Milroy, 1905; Dhéré, 1917].

3. *Compound of haematoporphyrin with copper.*

This substance was first prepared and described by Laidlaw [1904], who also proved that it is identical with or very closely allied to the pigment turacin [Church, 1869, 1892]. I think that the pigment prepared from the urine and described by Saillet [1896] as "iron free haemochromogen" was probably also this copper compound. Saillet obtained it by the action of ammonia on urinary haematoporphyrin. Recent work [C. Powell White] indicates that a minute trace of copper is present as a constant constituent of urine, and it, therefore, seems probable that the pigment was formed by the action of a trace of a copper salt on the ammoniacal solution of haematoporphyrin. I have repeated Saillet's experiment using pure haematoporphyrin without obtaining the "iron free haemochromogen."

The cupric compound can be easily prepared either in ammoniacal solution, or dissolved in glacial acetic acid. A $M/5000$ solution in glacial acetic acid was prepared in the following way. 0·002 g. of cupric acetate was added to 5 cc. of $M/1000$ haematoporphyrin dissolved in glacial acetic acid. 15 cc. of $N/10$ sodium acetate dissolved in glacial acetic acid were added, and the solution heated on the water-bath for about 20 minutes. The solution was then allowed to cool, and made up to 25 cc. with glacial acetic acid. The following absorption spectrum was found on examination.

Thickness of layer	Band I	Band II
25 mm.	$\underbrace{587{\cdot}8\text{–}580{\cdot}2}_{\text{Faint absorption}}\text{–}\underbrace{553{\cdot}3}_{\text{Intense absorption}}$	539·4–516·6
10 mm.	571–557·7	535–519·5

A $M/10,000$ solution of the copper compound in alcohol containing sulphuric acid of the same concentration as that used in the preparation of the similar solution of the nickel compound gave the following absorption spectrum.

Thickness of layer	Band I	Band II
25 mm.	572·5–553·3	538·4–517·12

Band II is much less intense and persistent than Band I.

The copper compound therefore resembles the nickel and cobalt derivatives in being stable in the presence of free mineral acids.

4. *Compound of haematoporphyrin with tin.*

Haematoporphyrin also forms a compound with tin which is characterised by two well-defined and persistent absorption bands, and by its great stability in mineral acid solutions.

A $M/5000$ solution of this pigment was prepared in the following way. 0·5 cc. of M stannous chloride was added to 5 cc. of $M/1000$ haematoporphyrin dissolved in glacial acetic acid, 8 cc. of N sodium acetate dissolved in glacial acetic acid were then added, the solution was heated for 20 minutes on the water-bath, then cooled, and made up to 25 cc. with glacial acetic acid. The resulting solution was filtered, and examined spectroscopically.

Thickness of layer	Band I	Band II
10 mm.	580·7–568·7	547·5–526·8
5 mm.	579·25–569·2	545·6–528·7

A $M/10{,}000$ solution of the stannous compound in alcohol containing sulphuric acid was prepared in the same way as the corresponding solutions of the other pigments and showed the following absorption spectrum.

Thickness of layer	Band I	Band II
25 mm.	580·7–568·2	547·5–525·4

The stannous compound can not only be prepared synthetically from haematoporphyrin, but also directly from haematin, or from defibrinated blood.

The following method was used for preparing the pigment from pure haematin. 4 cc. of a $M/400$ solution of pure haematin, prepared in the way previously described, were placed in a 50 cc. flask, 20 cc. of glacial acetic acid were added, and the solution was then heated on the water-bath for 20 minutes. 3 cc. of $2M$ stannous chloride were then added and the heating continued for one hour. On examining this solution with a small direct vision spectroscope, the absorption spectrum of acid haematoporphyrin was recognised. 15 cc. of N sodium acetate in glacial acetic acid were added, and the fluid was again heated for half an hour. The cooled solution was then made up to 50 cc. with glacial acetic acid. One portion of the solution was diluted with an equal volume of ethyl alcohol so as to form a $M/10{,}000$ solution. Another portion was diluted with an equal volume of alcohol containing sulphuric acid of the same concentration as that used in the previous preparations. The filtered solutions showed the following absorption spectra.

Nature of solution	Thickness of layer	Band I	Band II
M/10,000 Sn compound in acetic acid and alcohol	25 mm.	586–569·7	548–531·6
M/10,000 Sn compound in sulphuric acid and alcohol	25 mm.	587–570	552–530

It will be noticed that the absorption bands are situated nearer the red end of the spectrum than those of the synthetic pigment, and that no marked change is produced in the absorption spectrum by the action of sulphuric acid.

The absorption bands of the stannous compound are very persistent. A $M/250{,}000$ solution of the synthetic pigment still shows faintly the two characteristic absorption bands examined in a layer of 10 mm. with a direct vision spectroscope. Its absorption bands have a greater power of resisting dilution than those of haemochromogen. The more intense of the two absorption bands of a $M/100{,}000$ solution of pure haemochromogen, prepared by the reduction of haematin in alkaline solution with hydrazine hydrate could not be detected in a layer of 10 mm.

Stannous compound as a test for blood pigment.

The formation of the stannous compound can therefore be utilised as a very delicate test for blood pigment. The test may be rapidly carried out in the following way. Three drops of defibrinated ox blood are added to 5 cc. of glacial acetic acid placed in a test tube. The test may also be carried out equally well with much less blood, *e.g.* one drop of blood to 10 cc. of glacial acetic acid. The use of a larger amount of blood has the advantage of allowing one to trace clearly the intermediate stages in the formation of the stannous compound. The solution is then heated to the boiling point. It will be found to show the absorption spectrum of acid haematin. Three drops of $2M$ stannous chloride are added, and the solution is boiled for about one minute. When the precipitate, which forms, has been filtered off or allowed to settle, the absorption bands of acid haematoporphyrin can easily be seen on spectroscopic examination. A small quantity of solid sodium acetate is then added, and the solution again boiled. The fluid which has become brighter red in colour, is cooled and filtered. On spectroscopic examination of the filtrate the two characteristic absorption bands of the stannous derivative will be clearly seen. They closely resemble in position and character the bands of oxyhaemoglobin.

The application of the method to the examination of suspected stains for dried blood is very simple. A portion of the stained tissue is extracted by boiling with a little glacial acetic acid in a small test tube, and the solution of haematin thus obtained is treated in the way already described.

The following simple experiments illustrate the delicacy of the reaction as a test for blood pigment compared with the haemochromogen test.

1 cc. of diluted defibrinated ox blood (1 in 10 dilution in water) was added to 7 cc. of glacial acetic acid, boiled for a few minutes, mixed with four drops of $2M$ stannous chloride and again boiled gently for about five minutes. A small quantity of sodium acetate was added to the warm solution, which was then again boiled, allowed to cool, and finally made up to 10 cc. with glacial acetic acid; 5 cc. of this solution made up to 50 cc. with ethyl alcohol, and filtered, showed very distinctly the two absorption bands of the stannous derivative on spectroscopic examination. 5 cc. of the latter solution were diluted to 25 cc. and again filtered. The final stage of dilution therefore

corresponded to one volume of blood in 5000. When this solution was examined in a layer 25 mm. thick, the two absorption bands of the stannous compound could be seen, although they were faint.

The haemochromogen test was carried out for comparison in the following way.

10 cc. of a 1 in 1000 dilution of defibrinated ox blood were heated with 1 cc. of concentrated ammonia, then mixed with 5 cc. of ammonium sulphide solution, and made up to 20 cc. with water. Spectroscopic examination of this solution which corresponds with one volume of blood in 2000, showed one very faint absorption band. At a dilution of 1 in 5000, no absorption band could be detected in a layer of 25 mm.

A method for increasing the delicacy of the test.

After several other methods had been tried, the following method was found to give the most satisfactory results. Since known molecular concentrations of the various pigments have been dealt with throughout the greater part of this paper, it was considered advisable to work with solutions of oxyhaemoglobin of approximately known molecular concentration. Defibrinated ox blood having a specific gravity of 1050 was used for the test. Abderhalden's [1898] determinations of the iron as ferric oxide in ox blood were taken as a basis for calculation.

100 g. of ox blood contain 0·03805 g. of iron (calculated from Abderhalden's figures for ferric oxide). The ox blood used had a specific gravity of 1050 and 100 cc. of it therefore contained 0·03995 g. of iron; 139·8 cc. of ox blood would consequently contain 0·05584 g. of iron, and would be equivalent to 100 cc. of a $M/100$ solution of iron. 60·2 cc. of water were measured into a 200 cc. flask, and the solution was made up to 200 cc. with blood. The resulting solution is $M/200$ as regards its iron content, and, on the assumption that all the iron is present in combination as haemoglobin, the solution would also be approximately a $M/200$ solution of oxyhaemoglobin. 10 cc. of this solution were made up to 250 cc. with water. The resulting solution of oxyhaemoglobin was therefore $M/5000$. It may be, incidentally, of some interest to give the results of a spectroscopic examination of this solution compared with one of carbonic oxide haemoglobin of the same concentration.

Pigment	Thickness of layer	Band I	Band II
HbO_2	10 mm.	585 –567	550·7–527
HbCO	10 mm.	582·1–560	548·5–522·9

2·5 cc. of the $M/5000$ solution of oxyhaemoglobin were transferred to a 500 cc. flask, and made up to 500 cc. with water. The resulting solution of oxyhaemoglobin was therefore $M \times 10^{-6}$. 150 cc. of this solution were measured into a 250 cc. flask, 6·3 g. of pure re-distilled phenol were added, the fluid was gradually heated on the water-bath, and then kept at about 90° for 20 minutes. The solution was then saturated with ammonium sulphate (about 114 g.). Sodium chloride or sodium sulphate may be used instead of ammonium sulphate, but are less effective, the separation of the phenol being slower and apparently less complete, especially in the case of sodium chloride.

The phenol, after being thus "salted out," rose to the surface of the saline solution as a brown coloured layer containing the blood pigment in the form of haematin. Sufficient saturated solution of ammonium sulphate was added to the cooled fluid to bring the phenol solution entirely into the narrow neck of the flask. A small quantity of the phenol solution was transferred by means of a pipette to a test tube, filtered, mixed with half its volume of glacial acetic acid, and heated nearly to the boiling point. 4–6 drops of $2M$ stannous chloride were added and the solution was boiled for about two minutes, a few crystals of sodium acetate were added, and the solution was again boiled, and filtered. On examining the filtrate, placed in a test tube of about 11 mm. diameter, with the aid of a small direct vision spectroscope, the two characteristic absorption bands of the stannous compound were distinctly visible.

It is possible by means of this method to detect the presence of blood pigment in a concentration of $10^{-6} \times M/5$. In the latter case, 200 cc. of a $10^{-6} \times M/5$ solution of oxyhaemoglobin were placed in a 300 cc. flask, and 5·9 g. of phenol were added. The rest of the method was carried out in the same way as for the solution of higher concentration.

I believe that the foregoing test will be found to be the most delicate specific reaction for blood pigment as yet available, the guaiac, benzidine, phenolphthalin, and similar tests not yielding absolutely conclusive results.

Isolation of the stannous compound.

Attempts to isolate the stannous compound in a pure state have not been entirely successful. The following is a description of the method used in the preparation of the pigment. 10 g. of stannous chloride, 10 cc. of concentrated hydrochloric acid, and about 2 g. of pure finely granulated tin were heated with 100 cc. of glacial acetic acid in a round bottomed flask of one litre capacity until the greater part of the tin had dissolved. 100 cc. of glacial acetic acid and 25 g. of anhydrous sodium acetate were then added. While this solution was kept at 90°, 100 cc. of defibrinated ox blood were gradually added. The flask containing the solution was then closed with a rubber stopper provided with a glass tube and stopcock. The solution was allowed to cool to about 80°, the flask was evacuated, care being taken that none of the fluid was lost by frothing, the glass tap was turned so as to cut off the interior of the flask, and the flask together with the glass tap was detached from the aspirating pump. The object of heating the fluid *in vacuo* was to hinder re-oxidation of the reduced haematin by the oxygen of the air. The evacuated flask was then heated on the water-bath to about 80° for 45 minutes. The solution acquired a bright red colour. A small sample of the fluid after dilution with glacial acetic acid showed on spectroscopic examination the two characteristic absorption bands of the stannous compound.

On standing for 24 hours, a large proportion of the pigment was found to have separated out mainly in the amorphous form, but partly as small rhombic crystals of red colour. At first, it was hoped that the pigment might be separated from the mother liquid as in Schalfejeff's [1885] method for the preparation of haemin; but this proved to be impossible as the pigment settles very slowly from the solution. It also proved to be impossible to separate the pigment by means of the centrifuge. The jelly-like products of the action of the acetic acid on the proteins of the blood also render filtration impossible.

The following method was therefore adopted for freeing the pigment from the excess of stannous chloride, and from part of the proteins of the blood. The solution and suspension of the pigment in glacial acetic acid was concentrated nearly to dryness by distilling off the glacial acetic acid *in vacuo* on the water-bath. The residue was washed several times with water, saturated with potassium carbonate and extracted with ethyl alcohol. The alkaline alcoholic solution containing the greater part of the pigment was separated from the aqueous saline layer, filtered, and transferred to a distillation flask. The alcohol was then distilled off under reduced pressure. The pigment residue was dissolved in water, sufficient alkali being present to bring the greater part of it into solution, the liquid was then filtered, and the pigment was precipitated from the filtrate by the addition of dilute acetic acid. This precipitate was filtered off, washed with water, and dissolved in dilute sodium carbonate solution. The alkaline solution was then filtered, and the pigment again precipitated from the filtrate by the addition of dilute acetic acid. This precipitate was finally collected on filter paper, washed with water and dried *in vacuo* over sulphuric acid.

The pigment obtained in this way is insoluble in neutral acetone, ether, ethyl and amyl alcohols and chloroform. It dissolves in ether, ethyl and amyl alcohols, and chloroform acidified with acetic acid; but only dilute solutions can be thus obtained. It is practically insoluble in acetone containing about 2 per cent. of acetic acid; but dissolves slightly in acetone acidified with hydrochloric or trichloracetic acid. It is only slightly soluble in pyridine, and in mixtures of pyridine with ethyl alcohol, but is more readily soluble in ammoniacal alcohol. It is practically insoluble in aniline; but dissolves readily in phenol. The pigment is insoluble in dilute aqueous mineral and organic acids, but is readily soluble in dilute aqueous solutions of the fixed caustic alkalies, in ammonia, and in dilute sodium or potassium carbonate solutions.

Further experiments are being made with the object of improving the method of preparation; but are at present too incomplete to justify description.

Determinations of the ash yielded by the pigment after purification by separation from saturated solutions in acid acetone and ammoniacal alcohol have not yielded constant percentages. The quantities of pigment dealt with were small varying from 0·14 up to 0·2972 g., and little stress can therefore be laid on the results. The solubility of the pigment in the above organic solvents is so slight that relatively large quantities of the solvents must be used in the preparation of small amounts of the pigment. One result of these estimations may prove of some significance, namely the fact that the percentage of stannic oxide yielded by the pigment was never higher than half that, which one would anticipate on the hypothesis that in the formation of the pigment one atom of tin replaces one atom of iron in the haematin molecule.

The stannous pigment is so resistant to the action of mineral acids that it may be dissolved in concentrated sulphuric acid without undergoing any distinct change in its absorption spectrum. The results of spectroscopic examination of solutions of the pigment of unknown strength in concentrated sulphuric acid are given in the following columns:

Pigment dissolved in H_2SO_4	Thickness of layer	Band I	Band II
Solution (1)	20 mm.	592·1–562·4	551·4–519
Solution (2) (more dilute)	20 mm.	581·1–568·7	542·7–527·8

This pigment is therefore even more resistant to the action of mineral acids than haematin.

5. *Compound of haematoporphyrin with lead.*

This compound is only formed in alkaline solution, being decomposed by acetic as well as by mineral acids. It was prepared in the following way.

10 cc. of $M/1000$ alkaline solution of haematoporphyrin, placed in a 50 cc. flask, were mixed with 25 cc. $N/10$ caustic soda and 0·3 cc. of $N/2$ solution of lead acetate. The solution was then heated for a few minutes to the boiling point, cooled and made up to 50 cc. with $N/10$ caustic soda. The following is the absorption spectrum of the compound:

Thickness of layer	Band
10 mm.	600–578
25 mm.	609–570

The solution has a greenish brown colour, and shows only one band in the visible spectrum.

The above mentioned metals are the only ones which have been found to form compounds with haematoporphyrin with the exception of manganese [Zaleski, 1904]. No spectroscopic evidence was obtained of the formation of metallic compounds with aluminium, uranium, silver, or magnesium in either acetic acid or alkaline solutions. The pigment, which I described in an earlier paper as resulting from the reduction of haematin with aluminium in acetic acid solution, owed its formation to the presence of a trace of tin in the aluminium powder [1901]. It is not formed when pure aluminium is used.

A SIMPLE METHOD FOR PREPARING METALLIC COMPOUNDS OF HAEMATOPORPHYRIN.

It is necessary to use pure haematoporphyrin for the preparation of metallic derivatives of known concentration; but, as a considerable amount of labour is involved in their preparation, the following simple method has proved of some value in facilitating the preparation of these pigments, and thus rendering them more accessible for observation. The proteins of defibrinated blood were precipitated with alcohol, dried and finely powdered. The precipitate, while still moist with alcohol, may also be used. 10 g. or somewhat less of the powder were then extracted with about 100 cc. of 85 per cent. ethyl alcohol acidified with sulphuric acid (2 cc. of the concentrated acid in each 100 cc. of alcohol). The dark brown solution of haematin thus obtained was filtered, a trace of aluminium powder was added to the filtrate, and the fluid was heated to the boiling point on the water-bath for at most three minutes. The solution, which acquired a bright red colour, was then filtered. Aluminium was selected as a reducing agent instead of zinc, since it does not form a compound with haematoporphyrin. On more prolonged heating the haematoporphyrin undergoes reduction, the solution becomes brown in colour and shows an absorption band in a similar position to that of urobilin. It is therefore necessary to avoid more prolonged heating. A small portion of the filtrate was diluted with ethyl alcohol, filtered, and examined spectroscopically. It gave the absorption spectrum of haematoporphyrin in acid solution.

The greater part of the filtrate was then divided into two portions.

One of these was rendered alkaline with ammonia, and the precipitate of aluminium hydroxide, which formed, was filtered off. The alkaline filtrate, which on spectroscopic examination showed the bands of haematoporphyrin in alkaline solution, was divided into three portions. To one of these a trace of a copper salt, to another a zinc salt, and to a third a cadmium salt was added. The solutions were then heated on the water-bath for a few minutes, allowed to cool, diluted with alcohol, filtered, and examined with a spectroscope. They show the absorption spectra of the metallic compounds. The copper compound is formed very rapidly, the zinc and cadmium compounds more slowly. In the case of the latter two compounds it is advisable to allow their solutions to stand for some hours before diluting them with alcohol and examining them with the spectroscope.

Excess of finely powdered anhydrous potassium carbonate was gradually added to the other half of the acid solution. After each addition of potassium carbonate the evolution of carbon dioxide should be allowed to cease before more of the alkali is added. The fluid was then heated on the water-bath until the alcoholic solution became brown and reacted alkaline to litmus paper. After the alcoholic solution had been allowed to cool, it was separated from the aqueous saline layer, and filtered. A small portion after dilution with alcohol was found to give the absorption spectrum of alkaline haematoporphyrin.

Preparation of the lead compound. A considerable excess of N caustic soda was added to a small portion of the alcoholic solution, a few drops of a solution of lead hydroxide in caustic alkali were added, and the fluid was heated on the water-bath for a few minutes. The solution acquired a greenish tint and showed the absorption spectrum of the lead compound.

Preparation of the other metallic compounds. The remainder of the alkaline alcoholic solution was rendered acid by the addition of about one-fourth its volume of glacial acetic acid. Traces of zinc, copper and nickel acetates, cobaltous chloride, and stannous chloride were added to five separate portions of the acid solution. When chlorides or sulphates of the metals are added it is advisable also to add a little sodium acetate. The solutions were then heated on the water-bath for about 20 minutes, diluted with ethyl alcohol or glacial acetic acid, filtered, and examined with the spectroscope. They showed the absorption spectra of the corresponding metallic derivatives.

Preparation of synthetic haematin in acid solution. A portion of the above mentioned acid solution was further diluted with twice or thrice its volume of glacial acetic acid, a trace of ferric chloride and a trace of iron wire were added, the solution was heated to the boiling point and kept in the water-bath for at least 30 minutes. On spectroscopic examination it was found to show the absorption spectrum of synthetic haematin in acid solution.

COMPARATIVE RESISTANCE TO THE EFFECT OF DILUTION SHOWN BY THE ABSORPTION BANDS OF THE PIGMENTS.

Since the observations already made with the aid of the diffraction spectroscope only refer to solutions of one or two molar concentrations, and therefore leave the question of the relative intensity of the absorption bands practically untouched, it was considered essential to supplement them by determining approximately the concentrations at which the various absorption bands are just visible.

For this purpose, solutions of known molecular concentration were examined in layers of gradually diminished thickness until the absorption bands ceased to be visible. The solutions investigated were placed in a Baly's [1905, p. 413] adjustable cell, and examined with the aid of a prism spectroscope. The pigments were examined in the same order as those already investigated by means of the diffraction spectroscope. The wave lengths of the limits of certain additional absorption bands which became manifest in the red region of the spectrum are also recorded. The absorption bands previously defined are numbered in the same way as in the earlier part of this paper.

A $M/10{,}000$ alkaline solution of haematoporphyrin examined in a layer 37 mm. thick shows two bands in the red region in addition to those already described, (1) having the limits 680–665 and (2) having the limits 650–640. Six absorption bands are therefore visible at this concentration. When a $M/100{,}000$ alkaline solution of haematoporphyrin is examined, each of these bands just remains visible, when the thickness of the layer traversed by the light is not reduced beyond the limits stated in mm. given in the following columns:

$M/100{,}000$ ALKALINE SOLUTION OF HAEMATOPORPHYRIN.

Band (1)	Band (2)	Band 1	Band 2	Band 3	Band 4
120 mm.	55 mm.	37 mm.	22 mm.	15 mm.	15 mm.

The two most persistent absorption bands are therefore 3 and 4.

A $M/100,000$ solution of haematoporphyrin in alcohol acidified with sulphuric acid gave the following results:

Band 1	Band 2
13 mm.	8 mm.

Both bands of the pigment in acid solution are therefore more persistent than those of the pigment dissolved in alkali.

1. *Zinc and Cadmium compounds.* A $M/100,000$ solution of the zinc compound dissolved in alcohol acidified with acetic acid gave the following results:

Band 1	Band 2
11 mm.	11 mm.

The two bands are therefore equally persistent.

A $M/5000$ ammoniacal solution of the cadmium compound examined in a layer of 25 mm. shows two faint bands in the red region in addition to those already described. These bands have the following positions: (1) 645 and (2) 630–612. Their power of resisting dilution was not determined. When the solution was allowed to stand for some hours, bands (1) and (2) disappear. They were, therefore, apparently due to the presence of some unchanged haematoporphyrin.

A $M/50,000$ solution of the cadmium compound gave the following results:

Band 1	Band 2
32 mm.	30 mm.

2. *Nickel, Cobalt and Iron compounds.* $M/100,000$ solutions of the nickel and cobalt compounds gave the following results:

Pigment	Band 1	Band 2
Nickel compound	12 mm.	26 mm.
Cobalt compound	11 mm.	15 mm.

It will be noticed that in both cases bands 2 are less persistent than 1; but in the case of the cobalt compound they are of more nearly equal intensity.

The results for an alkaline solution of haemochromogen prepared from pure haematin by reduction with hydrazine hydrate are given below for comparison:

Pigment	Band 1	Band 2
$M/100,000$ haemochromogen	12 mm.	55 mm.

The difference in intensity of the two bands is more marked in this case than in those of the nickel and cobalt compounds.

Iron compounds. The characteristic absorption band in the red region given by solutions of haematin, both natural and synthetic, is much less persistent than the absorption bands of the foregoing pigments. The solutions examined were $M/25{,}000$; but the results are stated for $M/100{,}000$ concentration to secure uniformity.

Pigment	Band
Synthetic haematin	132 mm.
Natural haematin	160 mm.

The absorption band in the red of natural haematin is therefore less persistent than that of synthetic haematin. Both pigments were dissolved in alcohol acidified with acetic acid, the acid concentration being the same in both cases.

3. *Copper compound.* A $M/100{,}000$ solution of the copper compound dissolved in alcohol acidified with acetic acid gave the following results:

Band 1	Band 2
6 mm.	22 mm.

It will be noticed than Band 1 is more persistent than the absorption bands of any of the pigments hitherto examined. The absorption spectrum resembles those of the nickel and cobalt compounds and of haemochromogen in the marked difference in relative intensity of the bands.

4. *Stannous compound.* A preliminary examination of a $M/100{,}000$ solution of the stannous compound of haematoporphyrin dissolved in alcohol acidified with acetic acid proved that further dilution was necessary. A $M/500{,}000$ solution was therefore examined, and gave the following results:

Band 1	Band 2
10 mm.	11 mm.

The two bands therefore appear to offer practically the same resistance to dilution. The absorption bands have also a greater intensity than those of any of the other metallic compounds of haematoporphyrin.

When higher concentrations of the stannous compound of haematoporphyrin are examined with a prism spectroscope, an additional band is seen in the red region of the spectrum.

The following is the absorption spectrum of a $M/5000$ solution examined in a layer of 25 mm.

Band (1)	Band 1	Band 2
638–620	598–563	560–517

When a $M/25{,}000$ solution of the stannous compound is examined in a layer 10 mm. thick, Band (1) can just be detected. It ceases to be visible when the thickness of layer is reduced beyond this amount.

The foregoing results are obviously incomplete not only as regards the limitations of the methods employed, to which reference has already been made in the introduction; but also because the observations are limited to the visible spectrum.

The great stability of the stannous compound gives rise to the view, that it would be interesting to find out what metabolic changes, if any, it undergoes within the animal body after subcutaneous or intravenous injection. The great persistence of the bands in its absorption spectrum would facilitate its recognition in the organism after injection. Further, in view of the marked optical sensitising action which haematoporphyrin exercises on the animal organism [Pfeiffer, 1911, and others] the question arises whether the stannous compound or other stable metallic derivatives of haematoporphyrin have a similar action.

At a later date, I hope to carry out work on this subject along these lines.

SUMMARY.

1. The methods of preparation and the absorption spectra of the compounds of haematoporphyrin with zinc, cadmium, nickel, cobalt, iron, copper, tin and lead have been described.

2. The resistance of the pigments to the action of mineral acids has been determined.

3. A method for the direct preparation of the stannous compound from blood or haematin has been described.

4. A delicate test, based on the preparation of the stannous compound has been devised for the detection of traces of blood pigment.

5. A simple and rapid method for the preparation of metallic compounds of haematoporphyrin has been given.

6. The resistance of the absorption bands of the pigments to dilution has been approximately determined.

In conclusion, I should like to express my deep indebtedness to Professor W. B. Morton for lending me two spectrometers for use with grating and prism.

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XXVIII. THE PROTEINS OF COW'S COLOSTRUM. PART I. THE RELATION BETWEEN THE EUGLOBULIN AND PSEUDOGLOBULIN OF COW'S COLOSTRUM.

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In a previous communication [1915] the authors have brought forward data which demonstrate that the intramolecular arrangements of the amino-acids in the caseinogens of the cow and the sheep are not identical. This conclusion was arrived at from a comparison of the optical properties of the amino-acids derived from "racemised" cow's and sheep's caseinogens.

In subsequent work the method of protein racemisation has been extended successfully to the study of other proteins in an investigation which had for its object the elucidation of the relationships existing between the proteins of cow's colostrum and the corresponding compounds of ox-serum. In the present communication we give the results of the first part of this work, in which the relations between the globulins of cow's colostrum have been determined.

It has long been known that globulin, both in colostrum and in serum, consists of a mixture of two proteins, namely, euglobulin and pseudoglobulin. These compounds are characterised by widely different physical properties and solubilities. If a solution of globulin be submitted to dialysis, the euglobulin separates out, whilst the pseudoglobulin remains in solution.

The chemical relationship between the euglobulin and pseudoglobulin of serum has formed the subject of an investigation by Chick [1914], who concluded that the two proteins were very closely related. She found that pseudoglobulin preparations always contained, even at the end of a long dialysis, large quantities of a water-insoluble protein resembling euglobulin, and that the latter was largely derived from the pseudoglobulin by a gradual process of "denaturation." She suggested that this artificial euglobulin was a mechanical complex originating from the interaction and mutual precipitation of two colloidal systems, viz. pseudoglobulin solution and lipoid emulsion. It appeared probable that serum euglobulin was a protein-lipoid complex of similar origin.

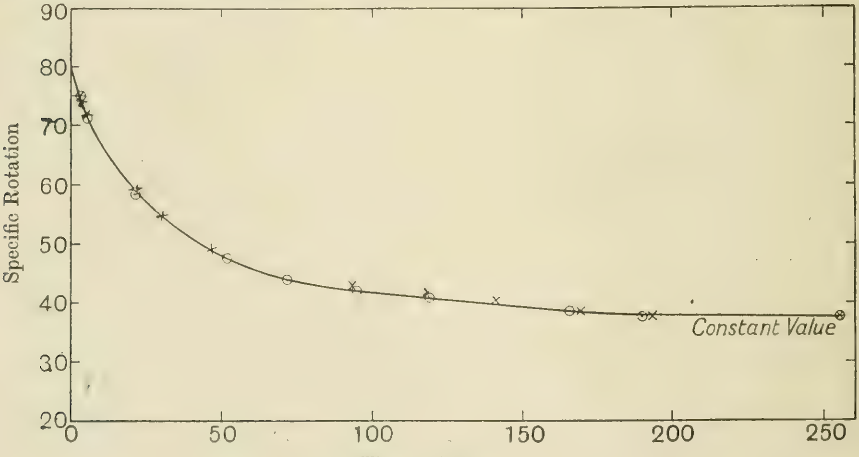
Further work by Hartley [1914] on this subject lends support to this view. The figures obtained by him in the analysis of serum euglobulin and pseudoglobulin by the method of van Slyke indicated that these substances were very similar in chemical composition.

Hitherto, little work of a comparative nature has been carried out with euglobulin and pseudoglobulin of cow's colostrum. In this paper we bring forward evidence in support of the view that the globulins of colostrum, just as of serum, are very closely related, if not actually identical, in chemical structure. Our experiments have indeed failed to reveal any structural differences at all between colostrum euglobulin and pseudoglobulin.

This conclusion has been arrived at by two independent methods of investigation, viz.

1. A comparison of the optical rotatory properties of euglobulin and pseudoglobulin in alkaline solution.

2. A comparative study of the optical properties of the amino-acids derived from the hydrolysis of "racemised" euglobulin and pseudoglobulin.



Graph showing progress of racemisation in $N/2$ alkali

If either of the globulins be dissolved in dilute alkali and the solution allowed to remain at 37° for several days, the initial rotation gradually diminishes until it reaches a practically constant value. The course of such a "racemisation" is represented graphically in the accompanying diagram.

The ordinates represent specific rotation values and the abscissae the number of hours the reaction had been allowed to proceed. The solutions were made in semi-normal sodium hydroxide and contained about 7 per cent. of protein (see experimental part).

The points indicated by small circles represent a series of specific rotations, determined on a solution of euglobulin in *N*/2 alkali (7·35 g. protein per 100 cc. solution) at different intervals during the period of racemisation. A second series of readings on a solution of pseudoglobulin in *N*/2 soda (7·04 g. protein per 100 cc. solution) is represented by the crosses. The diagram shows clearly that the optical behaviour of the globulins in *N*/2 alkali is identical. One curve can be drawn to satisfy equally both sets of points. Not only are the initial and final rotations the same in both cases, but the rate of diminution of the optical rotation, which in all probability is conditioned by intramolecular structure, is identical for both globulins.

If *N*/4 instead of *N*/2 alkali be employed as a solvent for the proteins, a rather different optical behaviour becomes apparent. It is found that in each case, using solutions containing about 7 per cent. protein, the specific rotation sinks from an initial rotation of about − 75° to a final constant value of about − 66°, as compared with a final value of about − 37° in *N*/2 alkali. In other words, the reaction does not proceed to anything like the same extent in *N*/4 alkali as it does in *N*/2 alkali. The significance of this difference is not quite clear as yet; it is possible, however, that this behaviour may indicate the existence of a definite stage in the racemisation process. Acidification at this stage causes the separation of a flocculent precipitate, whereas in the case where the stronger alkali has been employed, addition of dilute acid only occasions a very slight flocculation. It will be interesting to study in greater detail the process of racemisation under a variety of conditions and to note the influence of differing protein and alkali concentrations on the course of the reaction. For the present, however, it is sufficient to note that the behaviour of the two globulins in respect of their optical rotations is identical in *N*/4 just as it is in *N*/2 alkali.

An explanation of the behaviour of such protein solutions in suffering a gradual diminution of their specific rotations has been put forward by Dakin [1912]. According to this view, the racemisation of proteins by dilute alkali at low temperatures is due to a keto-enol tautomerism of the $>CH-CO-$ groups in the protein complex.

$$>CH-CO-NH- \longrightarrow >C=COH-NH-.$$

Wherever this change occurs, the asymmetry of that particular grouping will be destroyed. Consequently, if the racemised protein be hydrolysed, it will be found that certain of the amino-acids are totally inactive, whilst others are fully or partially active. It is extremely probable that the optical properties of the amino-acids thus isolated depend upon their positions in the peptide chains. Those amino-acid components of the protein molecule, whose carboxyl groups are free and which therefore may occupy terminal positions at the ends of peptide chains, will be incapable of racemisation, whilst those which are attached to the protein molecule by condensation of their carboxyl groups with the amino-groups of other constituents, and are therefore probably situated within the chains, may undergo racemisation, since with such an arrangement the keto-enol tautomeric change is possible, this change being accompanied by loss of asymmetry of the grouping in question. On the grounds of the absolute stability of racemised caseinogen and caseose against the common proteolytic enzymes, Dakin and Dudley [1913] concluded that the racemisation due to this tautomeric change must be complete for every such group affected in the protein complex. It thus becomes possible to obtain some evidence as to the original positions of the amino-acids within the protein molecule by a study of the optical properties of these acids among the hydrolytic products of the racemised protein.

In the accompanying table are given the optical properties of the amino-acids derived from the hydrolysis of racemised euglobulin and pseudoglobulin. The results obtained in a similar manner by the authors [1915] for the caseinogens of the cow and the sheep are also tabulated for purposes of comparison.

	Racemised colostrum euglobulin	Racemised colostrum pseudoglobulin	Racemised cow's caseinogen	Racemised sheep's caseinogen
Glycine... ...	—	—	—	—
Alanine ...	inactive and *d*	inactive and *d*	inactive (and *d*?)	inactive (and *d*?)
Valine	*d* and inactive	*d* and inactive	*d* and inactive	*d* and inactive
Leucine ...	*l* and inactive	*l* and inactive	*l* and inactive	*l* and inactive
Tyrosine ...	inactive	inactive	inactive	*l*
Phenylalanine ...	inactive	inactive	inactive	inactive
Proline	*l*	*l*	*l*	*l*
Aspartic acid ...	inactive	inactive	inactive	inactive
Glutaminic acid	*d* and inactive	*d* and inactive	inactive	*d* and inactive
Arginine ...	inactive	inactive	inactive	inactive
Histidine ...	inactive	inactive	inactive	inactive
Lysine	inactive	inactive	inactive	*d*

It will be noted that the amino-acids from racemised euglobulin and pseudoglobulin exhibit no differences whatever. This is regarded as a further confirmation of the structural identity of the two proteins.

The inactivity of the "hexone bases" is noteworthy; it would seem that these diamino-acids in the globulins (as also in cow's caseinogen) are situated entirely within the peptide chains. This fact would appear to lend support to the view sometimes put forward, that they represent the actual "foundation stones" on which the protein molecule is erected. Their basic character would naturally fit them for filling such a function.

It is also curious to notice that, though globulin contains about 3·5 per cent. of glycine, yet in neither case was this amino-acid found among the products of hydrolysis of the racemised globulins. This may possibly be due to the glycine being split off during the racemisation with loss of ammonia, since a certain amount of hydrolysis always occurs simultaneously with racemisation. If this be true, it would seem to indicate that the total glycine in globulin is so situated within the molecule, that it is split off with extreme ease by the action of hydrolysing agents. In this respect, globulin exhibits a markedly different behaviour from gelatin, as Dakin [1912] was able to recover about 17 per cent. of glycine from the products of hydrolysis of racemised gelatin. Eu- and pseudo-globulin behave identically in this respect; so that this fact is also consistent with the idea of their structural similarity.

In comparing the optical properties of the amino-acids derived from the hydrolysis of racemised cow's caseinogen and colostrum globulin, one is struck by the close similarity displayed, suggestive, it may be, of a near structural relationship between racemised caseinogen and racemised globulin. Such a kinship is not altogether impossible, in view of the fact that globulin appears to lose glycine during racemisation, whereas caseinogen, in like circumstances, suffers a loss of phosphate content.

In conclusion, reference may be made to an interesting confirmation of the results arrived at in this investigation, regarding the identity of the globulins in colostrum, which is furnished by work carried out in this Institution by Crowther and Raistrick [1916]. They analysed euglobulin and pseudo-globulin by the method of van Slyke and were unable to detect any differences in the amino-acid content of the two proteins. The results of their analyses demonstrated further that the total globulin of milk was identical in composition with that of colostrum.

EXPERIMENTAL.

Preparation of euglobulin and pseudoglobulin from cow's colostrum.

Colostrum, which is the fluid secreted by the mammary glands in the earliest stages after parturition, is eminently suited to be the starting point for the preparation of globulin. The colostrum secretion is distinguished by its richness in protein material, which may at times amount to as much as 18 to 20 per cent. of the total secretion. Of this, 4–5 per cent. usually consists of caseinogen, 0·7–1·5 per cent. of albumin and 6–12 per cent. of globulin. This is in striking contrast to the ordinary milk secretion which usually contains about 3·0 per cent caseinogen and 0·4 per cent. albumin, whilst globulin is only present in very small amounts. The preponderance of globulin in the initial secretion rapidly disappears with the development of mammary activity and the colostrum assumes the characteristic composition of milk often within about 48 hours of calving [Crowther and Raistrick, 1916].

The first step in the preparation of the globulins was to precipitate the caseinogen by means of dilute acetic acid. The best conditions for precipitation were found to be as follows.

The colostrum was added to dilute acetic acid containing 2 cc. of glacial acetic acid per litre; for every litre of colostrum, it was found advisable to use 2 litres of the dilute acid. The mixing was carried out in a tall glass jar and the liquid was stirred vigorously during the addition. The caseinogen, carrying along with it the bulk of the fatty material, immediately separated out.

After allowing the caseinogen precipitate to settle, the clear liquid, containing the globulins and albumin in solution, was siphoned off. A further quantity of globulin liquor was obtained by centrifuging the residual slime of caseinogen and fat.

The solution was next passed through a folded filter paper and made exactly neutral by the addition of the requisite amount of dilute soda. The large volume of liquid was then reduced somewhat by concentration in a vacuum pan at a temperature of 40–45°, and a sufficient quantity of magnesium sulphate was then stirred into the concentrated solution to ensure complete saturation. This effected the separation of the globulins, which were filtered off on a large Buchner funnel. The filtrate was preserved with a view to isolating the albumin for further work.

The mass of mixed globulins was dissolved in water and resalted out by means of magnesium sulphate, in order to render the separation from albumin more complete. The globulin so obtained was once more dissolved in the minimum amount of water and the solution was thoroughly dialysed against running tap water, in the initial stages, and finally against distilled water. During this process, the euglobulin separated out in a gelatinous form from the solution. The pseudoglobulin, which is soluble in distilled water, remained in solution.

The dilute solution of pseudoglobulin, after filtration, was concentrated *in vacuo* at a temperature of 40–45°, until a content of about 17 per cent. protein was attained. Emulsions of euglobulin (after thorough washing with distilled water) were prepared of a similar strength by stirring the protein into water containing a little common salt in solution. The advantages of working with such protein preparations, instead of employing the dried materials, were twofold. Firstly, we were enabled to avoid all processes which might have exerted a "denaturating" effect on the protein (*e.g.* excessive heat, treatment with alcohol, etc.). Secondly, it was possible, after carrying out a nitrogen estimation on such preparations, to make up quickly and without difficulty, solutions of any desired strength in alkali. This was a point of some importance, as it was required to determine the optical rotations of such solutions with as little delay as possible.

Rotatory properties of euglobulin and pseudoglobulin in dilute alkali.

If a 10 per cent. solution of euglobulin or pseudoglobulin be made in $N/2$ caustic soda solution, agglutination occurs almost immediately, the solution thickening to the consistency of a jelly. The jelly-like mass softens after 15 to 20 minutes; but this occurs much more readily if the flask containing the solution be placed in an incubator at 37°. In quite a short time, a clear solution of the globulin is obtained, which can be filtered without difficulty.

It will be noted that both globulins display the same physical changes accompanying their solution in dilute alkali.

In the following series of determinations, solutions of globulin of a 7–8 per cent. concentration in $N/2$ alkali were employed. At higher concentrations than this, it was found that the change from the jelly phase to the clear filterable solution was slower than desirable, in view of the importance of determining the rotation as soon as possible after making the solution.

The method of preparing such a solution was as follows. 10 cc. of $5N/2$ caustic soda solution were run into a 50 cc. graduated flask. After diluting this with distilled water, a measured amount of the globulin preparation, containing about 3·5 g. of protein, was added quickly and the bulk made up to the 50 cc. mark by the addition of distilled water. During the mixing process, the contents of the flask were shaken vigorously. The flask was then placed in the incubator, and after about two hours, the solution was filtered and the optical rotation determined in a decimetre tube. The exact concentration of the filtered solution was determined by a Kjeldahl estimation on 5 cc.

The rotation of the solution was determined at regular intervals, until it had attained a constant value. The two tables which follow give the results of comparative trials with the two globulins in $N/2$ caustic soda solution. It will be noted that the data, which have been plotted in the diagram already referred to, failed to reveal any differences in the structural make-up of the globulins. Such slight discrepancies as may exist fall well within the limits of experimental error. In both cases, the specific rotation fell rather rapidly in the first stages of racemisation; but the rate of diminution became slower later on, until finally the rotatory value became practically constant.

I. Pseudoglobulin (7·04 g. in 100 cc. $N/2$ alkaline solution).

Time (in hours)	Rotation in 1d tube	$[\alpha]_D = \frac{\text{Rotation}}{\text{Concentration}}$
2·25	−5·22	−74·15
3·5	−5·18	−73·57
5	−5·00	−71·02
22·5	−4·15	−58·95
30·5	−3·83	−54·40
47	−3·45	−49·00
94	−3·03	−43·04
120	−2·93	−41·62
142	−2·87	−40·77
170·5	−2·73	−38·78
194·5	−2·68	−38·07
215·5	−2·65	−37·64
262·5	−2·65	−37·64

II. Euglobulin (7·35 g. in 100 cc. $N/2$ alkaline solution).

Time (in hours)	Rotation in 1d tube	$[\alpha]_D = \frac{\text{Rotation}}{\text{Concentration}}$
2·5	−5·46	−74·28
5	−5·18	−70·48
22	−4·23	−57·55
52	−3·45	−46·94
72·5	−3·20	−43·54
95	−3·08	−41·91
120	−3·02	−41·09
167	−2·86	−38·91
190·5	−2·76	−37·55
336	−2·76	−37·55

Before leaving this part of the subject, it may be pointed out that the method outlined above should prove of value in future investigations, which may be designed with a view to establishing the identity or non-identity of related proteins. Its relative simplicity and the small amounts of material required are points in its favour. Its operation does not involve the length of time and the vast amount of labour, which are required by many of the ordinary methods of protein investigation. Moreover, it may be made a very exhaustive test; since if two proteins are to be pronounced structurally identical, then they must not only display the same optical behaviour under one set of conditions, but must continue to do so when the concentration of the protein and the strength of the alkaline solution are varied.

Isolation of Amino-acids.

For the purpose of isolating the amino-acids, 10 per cent. solutions of the globulins in $N/2$ NaOH were digested at 37° for 8–10 days. The rotation in a decimetre tube fell during this period from about − 7·5° to a constant value of about − 4°.

Tyrosine. 45 g. euglobulin and an equal weight of pseudoglobulin were racemised separately. The alkaline solutions were hydrolysed with hydrochloric acid, the solutions filtered and evaporated *in vacuo* at 45°. Water was added to the residues, and the solutions were once more evaporated under these conditions. This process was repeated until the bulk of the hydrochloric acid had been removed. The residues were subsequently dissolved in water, boiled with the addition of animal charcoal and filtered. After making the solutions slightly alkaline by means of ammonia, they were concentrated and the tyrosine was allowed to crystallise. The two specimens of crude tyrosine were filtered off, washed with a little hot glacial acetic acid and then dissolved in dilute hydrochloric acid. After treatment with animal charcoal, the solutions were neutralised with ammonia. On cooling, tyrosine separated out in characteristic form. The two samples were further repeatedly crystallised from slightly ammoniacal solutions, in order to remove completely traces of cystine. They were then dissolved in 4 per cent. hydrochloric acid and their optical rotations determined. They were both found to be inactive.

The mother liquor, from which the crude tyrosine had crystallised, was acidified with hydrochloric acid and added to the acid solution of mixed amino-acids for investigation by the esterification method.

Arginine, histidine and lysine were isolated by the method of Kossel and

Kutscher [1900]. For this purpose, 135 g. of euglobulin and 150 g. of pseudoglobulin were racemised separately. Arginine and histidine were in both instances inactive. The lysine fractions displayed definite *laevo* rotations when first isolated, owing to contamination with *l*-proline. It was found possible to remove the latter by fractional precipitation of the phosphotungstates, but a more convenient method was as follows. The copper salts of the mixture were prepared in the dry, powdered form. The latter preparation was then thoroughly extracted with absolute alcohol, in which the proline-copper salt was readily soluble. The residual insoluble lysine-copper salt was dissolved in water, acidified slightly with dilute sulphuric acid and decomposed by means of sulphuretted hydrogen. After such treatment, the lysine preparations from both sources were found to be totally inactive, whereas the fraction obtained from the decomposition of the proline-copper salt possessed a decided *laevo* rotation.

The large volume of acid solution, obtained in the above isolation of the diamino-acids after the filtration of the phosphotungstate precipitates, was made almost neutral by the addition of powdered commercial baryta. After filtering, the solution was evaporated to a small bulk in a vacuum pan at 45°, and the remaining sulphuric acid was removed quantitatively from the concentrated liquid by means of baryta. The solution thus obtained was acidified with hydrochloric acid and utilised to augment the yields in the investigation of the amino-acids by the esterification method.

200 g. of euglobulin, and 240 g. of pseudoglobulin, were racemised separately in the manner already described and then hydrolysed with hydrochloric acid for the amino-acid separation by the esterification method. To the acid solutions were added the solutions of amino-acids recovered from the tyrosine and diamino-acid separations. Levene's modification of Fischer's method was employed [Levene and van Slyke, 1909].

Glycine. As already mentioned, no glycine was found among the amino-acid preparations resulting from the hydrolysis of racemised euglobulin and pseudoglobulin. The fraction of crystals containing alanine and valine, where the glycine, if present, should occur, was fractionally crystallised, and the percentage of nitrogen in the more soluble portion of the crystals was determined. After carrying out this procedure repeatedly, the percentage of nitrogen increased to a constant value of 15·7 per cent., which is the nitrogen content of pure alanine, whereas that for glycine is the much higher value of 18·7 per cent.

The absence of glycine was confirmed by our inability to obtain crystals of the characteristic picrate from the alanine fractions by the method of Levene [1906].

Alanine. Specimens of pure *i*-alanine were obtained by fractional crystallisation from both globulin sources. In both cases, it was also found possible to recover other fractions containing alanine and valine, in which the rotation was *dextro.* They consisted of mixtures of *d*-alanine with *i*- and *d*-valine.

Valine was isolated in mixtures of *i*- and *d*-valine, containing as much as 70 per cent. of the active form, from both racemised globulins. In addition to this, specimens of *i*-valine were isolated in each case, and the *dextro* variety was found to occur in leucine-valine mixtures and in valine-alanine fractions.

Leucine. Both racemised globulins gave leucine in the pure state almost inactive, whilst in each instance, fractions of *l*-leucine containing only small amounts of *d*-valine were isolated.

Phenylalanine was isolated in the pure state. The specimens from both racemised globulins were inactive.

Proline. In both cases, the proline was strongly *laevo* rotatory, but displayed the partial racemisation which always occurs as a result of the process of separation. It was probable that in neither case had any racemisation taken place previous to hydrolysis.

Aspartic acid was isolated in the inactive form from racemised euglobulin, as well as from racemised pseudoglobulin. In neither case was any of the active variety detected.

Glutaminic acid. A specimen of glutaminic acid containing about 44 per cent. of the *dextro* variety was isolated from the hydrolytic products of racemised euglobulin. Racemised pseudoglobulin yielded an acid, which contained about 37 per cent. of the active form. These results were sufficiently close to be confirmatory of the identity of the two globulins, especially in view of the fact that a partial racemisation of glutaminic acid is brought about as a result of the hydrolysis and the subsequent process of isolation.

SUMMARY.

It has been shown that:

1. Euglobulin and pseudoglobulin of cow's colostrum display the same behaviour in respect of their optical properties, when dissolved in $N/2$ and $N/4$ alkali.

2. The optical properties of the amino-acids derived from the hydrolysis of racemised colostrum euglobulin are identical with those of the corresponding acids from hydrolysed racemised colostrum pseudoglobulin.

These results are in accord with the findings of Hartley [1914] and Chick [1914] on the corresponding globulins of ox-serum, and of Crowther and Raistrick [1916] on the colostrum globulins; they are confirmatory of the hypothesis, that the two globulins are identical, at least as far as the protein part of their molecules is concerned.

This reservation is necessary in view of the observations of Hardy [1905] and Haslam [1913] on the phosphorus content of globulin prepared from ox-serum. Haslam prepared pseudoglobulin free from phosphorus and showed that euglobulin contained about 0·1 per cent. of phosphorus, about half of which was extractable with alcohol and ether.

The results given by Hartley on the one hand, and Crowther and Raistrick on the other, amply demonstrate that such slight differences are limited to the non-protein ingredients of the globulin preparations. Chick [1914], as a result of her investigations on the serum globulins, expressed the opinion that the phosphorus content of euglobulin was due to the association of the protein with a serum-lipoid. The incomplete extraction of the fatty substance containing the phosphorus in the euglobulin preparation was probably due to the state of aggregation of the protein and not to the phosphorus constituting an integral part of the euglobulin protein molecule.

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XXIX. ON THE THEORY OF GELS.

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In the present paper it is proposed to develop the theory, previously outlined [1917, 1, 2], that the gelation of the natural emulsoids is a crystallisation process, which follows from von Weimarn's theory in consequence of their small coefficient of diffusion and the considerable viscosity and molecular aggregation of their sols. The properties of gels result simply from the structure thus involved. In certain cases, such as the so-called thermal anomaly of gels, and the heat of imbibition of gelatin, where the recorded facts are at variance with the theory, previous observations have been shown to be in error and results obtained in agreement with the values required.

1. On the reversible sol-gel transformation.

Perhaps the theory of gel structure which has found most favour of late is that adopted by W. Ostwald which considers gels to be composed of two liquid phases persisting from the sol stage. Hatschek's mathematical investigation [1917] showed, from the stress-elongation curve, that this hypothesis is untenable. The assumption is implied that gels have a solid disperse phase, consisting of discrete particles, or of aggregations of such particles. We thus return to the ideas comprehended in the earliest theories of gel structure. M. L. Frankenheim [1835] considered that gels were composed of aggregates of small crystals with pores between, which might account for the low density, easy solubility and elasticity of such bodies. K. von Nägeli [1858] thought that a distinction should be made between distensible substances like gels and merely porous bodies. He supposed that gels consisted of molecular complexes or micellae with crystalline properties, separated by skins of water and forming meshes (or interstices) in which the water was contained by molecular attraction. Later [1862, 1] he wrote "Organised substances are composed of crystalline doubly refractive molecules aggregated from many atoms but which lie closely in definite arrangement with one another. In the moist condition, in consequence of the unbalanced attraction, each is surrounded with a skin of water." It has recently been suggested by more than one writer that this theory agrees most nearly with the properties of gels, and further study fully confirms this view. Although von Nägeli used the term "meshes (Maschen)" he may have referred merely to the interstices in the micellae. This appears probable in the light of a quotation given below. However, from this time, a number of unsuccessful attempts have been made to devise a geometrical framework which would account for the properties of gels. O. Bütschli's extensive researches [1892, 1896, 1898, 1900] on foams and gel structure are well known. As a result of microscopic examination he came to the opinion that the properties of gels might be explained upon the basis of a honeycomb structure in which the walls were permeable to liquids because of their extreme thinness, although he admitted that the walls might be porous.

W. Pauli [1902] objected to Bütschli's assumption that the use of alcohol and chromic acid in his experiments to make the microstructure visible did not alter the structure of the gels. Von Weimarn [1914] also pointed out that the honeycomb appearance is probably an optical effect due to the overlapping of grains. From a study of imbibition Arisz [1915] came to a decision adverse to a honeycomb structure.

Moreover, if such a structure is dependent on the directive forces of crystallisation, a different arrangement would seem to be implied for each gel. Such differences in the structure of microscopically visible crystal aggregates were indeed found by Zsigmondy and Bachmann [1912] in their work on the gelation of sodium and potassium oleate, palmitate and stearate sols. It seems probable therefore that a single network will not account for the elastic and thermal properties of different gels.

The hypothesis of a network is not supported by the viscous behaviour of gels which gradually mould themselves to the shape of the containing vessel. The gradual motion of small solid masses through gels, under the influence of gravity or of diffusing streams of particles, leaving the gel apparently unchanged, has frequently been noticed during recent work. Moreover suspended cylinders tended to become ellipsoidal.

Further evidence against a net structure is afforded by the lens-shaped form of gas bubbles generated in gels. This phenomenon was investigated by Hatschek [1914] who found that the bubbles place themselves at right angles to pressure and parallel to tension applied to the gel. By measuring the angles between pairs of bubbles in 10 per cent. gelatin, Hatschek found certain values approximately repeated a number of times, although other measurements varied enormously. A similar appearance may be obtained in 1 per cent. agar by immersing the free gel containing $N/5$ Na_2CO_3 in $N/4$ CH_3CO_2H. Planes of cleavage are clearly suggested. The simplest structure that would conform approximately to the conditions is that of piled shot. This agrees with the globulitic structure observed ultramicroscopically by Bachmann [1911]. Examination of gelatin gels of from 1–6 per cent., from which the liquid had been expressed, showed irregular flocks of separate granules with clear spaces intervening. Increasing concentration reduced the size of the grains (as would be required by von Weimarn's theory) and, at the same time, the empty spaces gradually disappeared, until, at about 6 per cent., the solid phase filled the whole field and the single granules could be no longer differentiated. Agar and silicic acid gels showed a similar globulitic structure.

From these considerations it seems probable that the solid phase of gels consists of discrete granular particles. Since it is now recognised that the distinction between the suspensoids and emulsoids is rather a question of the affinity between the two phases than of the solid or liquid state of the disperse phase, there seems to be the less reason for refusing to extend von Weimarn's theory to the natural emulsoids and regard their gelation as a crystallisation process.

A connection between the precipitation of colloids and the process of crystallisation is certainly indicated by the fact that both suspensoids and emulsoids exhibit the phenomenon of supersaturation. W. Ostwald [1897] has shown that there is a minimum quantity of a substance which is capable of causing the crystallisation of a metastable solution. In the case of sodium chlorate this is equal to about 10^{-10} g., corresponding to a cube with a side of several microns. This is well above the size of the disperse phase in sols. The existence of sols of greater concentration than the ordinary solubility of the substance concerned is therefore in harmony with the theory of supersaturation. The phenomena of supersaturation have frequently been observed in the case of sols. Thus gold separates spontaneously from gold sols of more than a certain concentration and the precipitation may be accelerated by adding a few drops of a second solution of colloid gold. Garrett [1903] found that gelatin sols gelate more rapidly if some solid gelatin, prepared by rapidly cooling the same sol, be added. The spontaneous coagulation of rubber emulsions is hastened by the addition of solid rubber. Additional evidence is furnished by the temperature variation of the concentration of the liquid expressed from gels. In the liquid from a 2·3 per cent. agar gel

Hardy [1900] found from 0·09 per cent. of agar at 5° to 0·47 per cent. at 36°. This is corroborated by the influence of concentration on the temperature of gelation [Pauli and Rona, 1902; Rohloff, 1907; Levites, 1908].

The literature of organic chemistry abounds in descriptions of definite substances which occur in the gel form. Frequently, by suitable choice of solvent, these have also been obtained as well defined crystals. The following examples are taken at random. The sodium salt of acrylic acid occurs as microscopic lenticular needles. The boiling saturated aqueous solution sets on cooling to a jelly which shows no structure under the microscope [Linnemann, 1874]. Glycyrrhizic acid, $C_{44}H_{63}O_{18}N$, which occurs in licorice root, crystallises from alcohol as light yellow glistening leaflets, which are the better formed the slower the separation from alcohol. It is characteristically little soluble in water at ordinary temperatures. In boiling water the substance is easily soluble and the solution forms on cooling a light wine coloured jelly. By mixing 1 g. of glycyrrhizic acid with 100 cc. of water a jelly is formed, after some hours standing, of such a consistency that the vessel may be inverted without loss [Habermann, 1879].

The gels of camphorylphenylthiosemicarbazide, investigated by Hatschek [1912], led him to suspect a crystalline structure formed by growth of amicrons into a network: a conclusion which he hesitated to accept on account of the mathematical difficulties. These gels appear to be typically elastic, and are produced (1) when a hot alcoholic solution of the substance up to about 5 per cent. is suddenly cooled, (2) from the gradual or rapid cooling of toluene solutions of similar concentration, or (3) by pouring an alcoholic solution into petroleum. The latter method gives gels with a concentration as small as 1 : 400. The solubilities in alcohol, toluene and petroleum are respectively 0·5 per cent., 1 per cent. and very small. Von Weimarn's theory affords a ready explanation of such cases. From the examination of a very large number of precipitates von Weimarn showed that the degree of dispersity in which a substance occurs is regulated by the number of crystallisation centres, N, produced according to the formula $N = KP/L$, where L is the solubility of the substance, P the excess concentration $(C - L)$, and K is a factor which depends on the degree of aggregation of the reaction components in solution and the viscosity of the reaction medium. A sol or gel is formed according as a large value of N is produced from a small value of L or a large value of P. Intermediate values of L and P give rise to micro or macro crystals. By actual inoculation of supersaturated solutions, von Weimarn has shown that in every stage, except the two extremes, the solid phase is crystalline.

He infers that the disperse phase of such sols and gels is likewise crystalline though the particles are too small to act as nuclei.

Considering the general case of a substance of high molecular weight and very low diffusivity, let this be treated with a liquid in which its solubility, L, shall be small in the cold and increase considerably, but not too rapidly on heating. Then, on applying heat, the process will be reversible and the Noyes-Nernst formula for the velocity of solution, $V = \frac{D}{\delta} S (L - C)$, will hold. Since L is very small, V will be very small indeed, and the sol stage will persist. Further heating will lead to the gradual breaking up of the aggregated particles of the sol and an approach to true solution. If the stage of single molecules is reached, this may be regarded as a true solution, although the diffusion constant will be that of a sol. Notwithstanding the size of the molecules, the system may be looked upon as homogeneous and von Weimarn's formula applied. On cooling, with reduction of L, aggregation will commence, but the velocity of crystallisation, $V = \frac{D}{\delta} S (C - L)$, will be a minimum, so that diminution of L will give a larger and larger P and a still greater value of N, since $\frac{dN}{dL} = - K \frac{C}{L^2}$. The velocity of crystallisation will also increase, and, if the laws of supersaturation apply, spontaneous crystallisation will eventually take place. Since N must necessarily be very large, from the large values of P and K, as well as the small value of L, a gel will be formed. Owing to the small velocity of crystallisation, gelation will however take place slowly. The formation of a gel will therefore be dependent on the concentration, the thermal variation of solubility and the factor K, which represents the degree of association and viscosity of the sol.

The natural emulsoids such as gelatin and agar appear to differ from the organic substances considered above, merely in the fact that they do not ordinarily occur in the crystalline form, though von Weimarn [1910] indeed claims to have obtained crystals of gelatin. Although little is known as to the true solubilities of these substances, there seems to be no reason for regarding them as exceptions to the general case. Actually their known properties are exactly such as to bring them within its scope. They form sols of considerable concentration, aggregation and viscosity. Heating at constant temperature decreases their viscosity, while cooling increases it [Levites, 1908], changes which are due to variation in the number as well as the size of the disperse particles and are thus seen to be in accordance with Einstein's formula. Osmotic pressure varies in the opposite sense.

The heat coagulation of albumin may be explained on the supposition of the chemical production of an insoluble compound. In this case the gelation should be, and actually is, irreversible. Silicic acid behaves very much in the same way as the typical case of aluminium hydroxide investigated by von Weimarn. The gel is formed immediately when the equivalent amount of hydrochloric acid is mixed with a moderately concentrated solution of potassium silicate. This is due to the sudden production of a highly aggregated insoluble form of silicic acid, owing to the concentration, aggregation and viscosity of the reaction medium. When, however, the silicate solution is slowly stirred into the mineral acid, thus keeping down the value of K, probably a true solution of silicic acid is formed at first, as is indicated by its high diffusivity.

While the foregoing cannot be considered as a rigid proof of the hypothesis, it seems permissible, in the absence of evidence to the contrary, to regard the reversible sol-gel transformation of the natural emulsoids as due to their large value of K, together with their low diffusion constant, which, by retarding crystallisation, allows the accumulation of a sufficiently large excess concentration to cause gelation on cooling. The theory receives confirmation from the fact that all the properties of gels are found to be consistent with the structure thus conditioned, and from the verification of certain new facts indicated thereby.

Similar considerations would appear to account for the occurrence also of resins and of glass. Indeed, from experiments on the introduction of metals into the surface layer of glass, Rhemberg is convinced that we should regard this as a substance full of ultramicroscopic pores. Various definite organic substances are easily obtained in either the crystalline or resinous form at will. Piperine crystallises from alcohol in monoclinic prisms which melt at 132° and set on cooling to a light yellow transparent resin. On heating to 100° the resin loses its transparency, becoming nearly opaque, like sulphur, owing to its transformation into a mass of microscopic crystals which undergo no further change until they melt at 132°. On cooling the resin is formed as before. If this is kept for about ten days, yellow sulphur-like spots of microscopic crystals begin to appear and very gradually extend throughout the mass. But after heating to 180° for an hour, this change does not begin in 2½ years [Madan, 1901].

2. On spherites in relation to gel structure.

Before proceeding to the examination of the properties of gels, it seems worth while to inquire into the actual form of the disperse phase. As mentioned above, ultramicroscopic evidence shows a globulitic structure, but since the specific surface of gels must be extremely large, it seems probable that the individual grains are themselves aggregated. Von Nägeli was unable to say whether his micellae were identical with spherites[1], but the evidence now available points to this conclusion. W. Ostwald [1891] states that microscopic observation of the process of crystallisation shows that (at least in the case of substances of low melting point) small spherical bodies first appear. Only after they have attained a certain size do they assume crystalline shape. Linck [1839] first observed that substances deposited from solution appear originally as small droplets. Frankenheim [1860] describes the appearance of globules in a drop of saturated aluminium sulphate in water made turbid by addition of alcohol. However, Richards and Archibald's cinematographic study [1901] of the first appearance of growing crystals failed to show the occurrence of globules in any of the cases investigated, and it is doubtful whether a transitory spherical phase appears, except in the case of substances of low melting point.

Spherites were first studied by Brewster [1853] who observed their production when solutions were allowed to crystallise under pressure between glass plates. He believed the pressure to be an essential cause of the phenomenon, but noticed the contributing effects of quick cooling and increase of viscosity. Rainey [1858] obtained microscopic spherites of calcite by allowing the slow mixing by diffusion of solutions of $CaCl_2$ and Na_2CO_3 in gum water. Kuhlmann, in 1863, pointed out the important influence of increasing the viscosity of the solution. With a view to slowing the crystallisation, he added such substances as gum and gelatin. When crystallisation took place sufficiently rapidly, the crystal particles which first appeared quickly grew into spherites. The structure of such bodies, occurring naturally in Acetabularia, was described by von Nägeli [1862, 2]. "The smallest spherites (up to about 40μ) were perfectly spherical.... In the unchanged state... they appear almost like oil drops or starch grains, though more glass like.... Often they show clear banding. The bands have a very regular and quite concentric relation to the surface.... This stratified arrangement proves that the spherites grow by additions to their surface....They appear as clear stripes which are mostly at exactly equal distances apart. Besides the concentric bands, frequently there appears a radial striation....The substance is divided thereby into meshes of more or less quadratic form....The ringed structure of the spherites is very like that of starch grains and cell membranes....By drying they become dark, since all their tiny meshes become filled with air." Von Nägeli also observed the beautiful brushes and rings which these crystal aggregates show in polarised light. Frankenheim remarks on the periodic growth of the spherites, which he considers the cause of the concentric rings like the yearly rings observed in a sawn tree stem. "After a time the process becomes slower, sometimes part will redissolve, but soon the exhausted liquid recovers itself, and growth begins anew, soon again to be interrupted." Frankenheim also recognised the relation between the concentric zonal structure frequently observed in minerals and the periodic growth of spherites. Dallas [1875] obtained beautiful spherites, especially of copper sulphate, lead acetate and morphine hydrochloride, by spreading the solution on glass plates and allowing rapid evaporation. Vogelsang [1875] studied the crystallisation of sulphur in carbon disulphide containing thickening material. The substance first appeared in the form of supersaturated droplets as Frankenheim had observed. These gave up more and more of the contained liquid and gradually solidified to isotropic spheres, or spherites. Reinisch, in 1884, obtained magnificent results with double salts of ammonium and sodium, prepared by dissolving 1 g. of ammonium sulphate and 2 g. of crystallised sodium sulphate in 80 parts of water and adding 10 per cent. of nitric acid. With a magnification of 20 the spheres were 2 cm. in diameter. They consisted of needles arranged radially with a notched edge, showed a grey shadow cross and three wide concentric colour rings, but only as long as the crystals were still moist. After drying the crystals appeared silvery white. Very beautiful results were obtained with erbium yttrium sulphate without the addition of nitric acid.

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[1] In this paper the term "spherite" is used to include spherical aggregates, spherical crystals, spherocrystals, spherulites, globulites and all such approximately spherical crystalline aggregations, the origin and structure of which appears to be similar.

Numberless examples are known of organic compounds which crystallise as spherites. These are usually substances of high molecular weight, or such as are probably much aggregated in solution. Inulin is deposited from water as spherites only when a saturated solution is slowly crystallised. Quick evaporation forms amorphous crusts. Evaporation of a drop of an aqueous solution on glass leaves a transparent horny fibrous mass. If the evaporation is slowed, as by placing a coverglass with a hanging drop of the inulin solution upon a moist cardboard frame, good doubly refracting spherites are obtained

[Leitgeb, 1888; Fischer, 1902]. The growth of spherites of amylodextrin was observed by Syniewski [1902]. "If a drop of the [formaldehyde] solution is placed on a microscope slide, covered with a cover glass, and brought under the microscope, after an hour the commencement of the crystallisation is seen. Needle-like crystals shoot at first to grouped star-like bundles. After a time these bundles become composed of regular concentrically arranged layers of extraordinarily fine needles directed towards the centre, which then show a thousand structures like starch grains. This imitation of starch grains goes so far that aggregated grains are also seen to originate. All these grains are composed of doubly refracting needles and show beautifully, in polarised light, the well known dark cross of the starch grains." The same author has shown that starch grains consist wholly of a single substance and that the amylocellulose, of which the outer skins of the grains were supposed to be formed, is a decomposition product. Having regard to Bütschli's [1898] preparation of artificial starch grains by crystallisation of starch in presence of gelatin, little doubt remains that the starch grains are themselves identical in origin and structure with spherites.

The production of large crystals by slow diffusion, as through strips of filter paper [Monnier, 1874], or gels [Vesque, 1874], is well known. In 1879 Ord observed the precipitation of calcium oxalate by diffusion of ammonium oxalate through an isinglass gel in an open tube immersed in calcium chloride solution. The precipitate consisted first of growth forms changing into spherites towards the calcium chloride, which must have been hypotonic. The very frequent occurrence of spherites in gels was pointed out by Hatschek and is being studied by the writer. Spherites of various difficultly soluble inorganic salts have been prepared by the diffusion of reagents into gels containing solutes with which they react. Frequently the precipitate formed in the upper regions of the gel, where the reagents are more concentrated, is composed of small needles or tiny regular crystals. As the dilution of the reagents increases and the velocity of crystallisation diminishes, the particles become much aggregated and approach spherical shape, often becoming perfect spheres in the lower nearly exhausted regions of the gel. Among others, good examples of spherites of silver bichromate, copper and calcium carbonates have been obtained. The former are opaque, the two latter substances show black crosses in polarised light, and the larger spheres colour rings. Spherites of manganese polysulphide have been produced over 1 cm. in diameter. These are opaque, but have the same banded structure. They resemble closely the large spheres, several inches in diameter, of magnesian limestone occurring in the Permian beds at Fulwell Hill, Sunderland, described by Abbott. These results show that the formation of spherites is not induced by quick crystallisation. In all the cases quoted it is, however, reasonable to assume a state of molecular aggregation in solution. The slow diffusion in the presence of protective colloid in gels favours the formation of such molecular aggregates in solution. Supersaturation, especially in viscous media and with bodies of high molecular weight, has the same effect, and since the crystallisation of a substance usually begins from a supersaturated solution, the first appearance of spherical masses would be in accordance with an existing state of aggregation. Consequently it may be doubted whether the "droplets" frequently recorded are invariably liquid. If they were so, the cause of their further development into spherites, instead of crystals, in some cases, would still require explanation. There are, however, many bodies of low melting point which undoubtedly appear at first in the form of liquid drops. From Hardy's [1900] experiments with gelatin sols treated with alcohol, this may possibly be the case in the gelation of gelatin. In accordance with Masson's [1891] theory of solution, gelatin may melt in water at a lower temperature than that of complete miscibility, below which there would exist two phases, one of melted solid containing a little water, and the other of water saturated with gelatin. The velocity of crystallisation of gelatin is so slow that even the melted solid phase may form spherites. In any case aggregation in solution appears to be a principal cause of the formation of spherites. Another important factor must be the viscosity of the liquid medium, which restricts the movement of the unwieldy molecules or molecular aggregates. Under the influence of the feebler forces of crystallisation, consequent upon the size of the particles, these have the less opportunity to pack themselves into regular crystals.

From the rapidity of the formation of the layers, Leitgeb [1888] has shown that they cannot be influenced by outside causes, but must be due to changes in the concentration of the solution in the immediate neighbourhood of the crystals. Observation of banded precipitates formed by diffusion of reagents into gels, indicates that the zonal structure is due to adsorption, by the precipitate, of solute from the gel, until the region next the precipitate is exhausted of solute, and the reagent is able to diffuse further into the gel before forming a fresh band [Bradford, 1916, 1, 2]. A similar explanation may be given of the zonal structure of spherites. Since these consist of numerous ultramicroscopic individual particles, their specific surface will be very large. Consequently the solute will be rapidly adsorbed and the concentration in the region next the spherite reduced. If the concentration fall sufficiently, crystallisation will be momentarily suspended or its velocity reduced until fresh supplies of nutrient material arrive.

These considerations lead to the conclusion that substances of high molecular weight and such as exist in solution in the aggregated form, tend to crystallise in the form of spherites. It is reasonable to suppose, therefore, that the granules seen ultramicroscopically in gels, which must have great specific surface, actually consist of these spherical aggregates.

Spherites of gelatin can easily be produced of microscopic size. A sample of dialysed gelatin precipitated by alcohol, was dissolved in about 100 parts of water and poured slowly into alcohol. The smallest trace of the precipitate brought on to a microscopic slide, showed a mass of innumerable transparent granules, round the edge of which individual grains could be seen. Under a magnification of about 800 the granules appeared perfectly spherical and translucent of about 0·5 to 2 mm. diameter (Fig. 1). No connection between the grains could be seen.

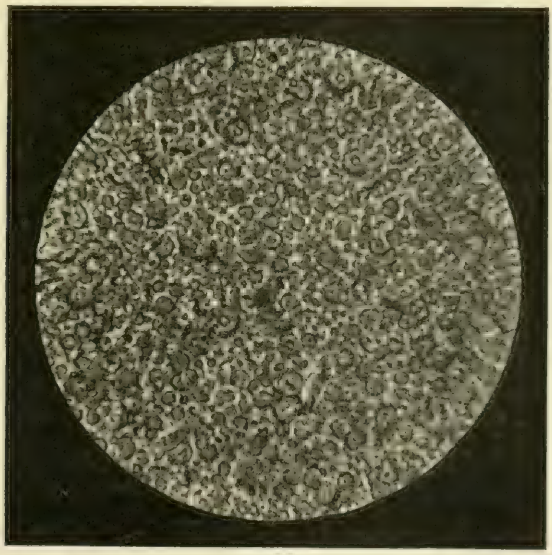

Fig. 1. Gelatin precipitated by alcohol ×800.

The question next arises as to whether the micellae are themselves aggregated to form structures within the gel. On account of the small size of the micellae, the problem is the more difficult to solve. Ultramicroscopic examination of untreated gels gives no indication of such aggregation.

Moore [1915] observed chains of globules in silicic acid sols. The possibility of the existence of microscopic filaments has been suggested [Bradford, 1917, 2]. This is not supported by the viscous properties of gels which permit the movement of solid particles and gas bubbles within them. Aggregation of the micellae is possibly more likely to occur in the surface skins of gels owing to the influence of adsorption and evaporation in increasing the concentration. Hitherto the influence of these skins on the properties of gels does not seem to have received sufficient attention. Moreover, if one region of a sol should gelate before another, adsorption might cause a rhythmic variation in the density of distribution of the gel particles similarly to the bands in spherites. The possibility of aggregation of the micellae within the gel has been investigated ultramicroscopically by Moeller [1916, 1917], who considers that the micellae are united into fibrils, like those in organic membranes, but which are obscured by the thickness of the layers viewed ultramicroscopically. To overcome this difficulty he observes very thin layers of gelating sol upon a microscope slide with a cardioid ultracondenser, and, before gelation, he adds alcohol or some tanning material. In other cases the surface of the gel is treated with the tanning solution, and the appearance observed before diffusion has reached the interior of the gel. These methods are not very different from those employed by Bütschli and others for microscopic examination. Unfortunately both methods are open to great objection. In the first case the addition of precipitating reagents to the sol fundamentally transforms the character of the crystallisation process, and it may be observed that the structures seen were immediately broken up by slightly disturbing the slide during gelation. In the second case, as has been shown, the properties of the surfaces of gels are likely to differ from those of the interior. In both cases the formation of precipitates by the reaction of the tanning reagents, such as chrome alum, with the impurities of the nature of calcium salts in the gel does not appear to be excluded. The presence of such impurities in the gelatin used is confirmed by a photograph showing spherites, produced in the drying gel, exactly resembling the form in which calcium sulphate occurs in gelatin. Such precipitates would undoubtedly be produced in any but the most carefully purified gelatin and might show structures similar to those observed. Moreover, it seems probable, as Wilson has pointed out, that gelatin forms insoluble compounds with alum and chrome tanning material. These would tend to be precipitated in the banded form. The production of similar laminated or striated forms by the crystallisation of salts in gelatin films, is well known, and originates from the action of adsorption and diffusion as in the Liesegang phenomenon [Bradford, 1916, 1, 2]. From the similarity of the structures seen with Liesegang's rings, Moeller argues, in the second paper, that the latter are due to the pre-existence of laminated structures in the gel. This is certainly controverted by many facts: such as that the distance apart and the width of the precipitated bands can be determined, in a given gel, by a suitable choice of the concentrations of the reacting solutions; that the orientation of the bands is always at right angles to the diffusion path; and that similar banding can be produced in sand columns and filter paper. Moeller also obtains various shrinkage figures, like those previously studied by Liesegang and others, in the surface of gelatin gels by the action of water vapour, hot water, or drying. From these experiments Moeller concludes that, of the α- and β-gelatin of which the gels are composed, the α-gelatin forms fibrils which intertwine to form a network, in the meshes of which is enclosed the β-gelatin, or gelatose, as a structureless mass in solution. The theory is then extended to include all gels. While however the possible existence of fibrils in the interior of gels cannot be definitely excluded at present, it may be considered that the available evidence is insufficient to establish their presence.

3. The structure and properties of gels.

From the point of view of the foregoing, the natural emulsoid gels may be regarded as two phase systems comprising innumerable ultramicroscopic globular crystalline molecular aggregates, or probably spherites, in, and around, which the mother liquor, from which the disperse phase has crystallised, is held by molecular and capillary forces.

Owing to the enormous specific surface, the state of a portion of the liquid phase will differ from that of a free liquid. Assuming with Laplace that the surface tension and intrinsic pressure of liquids may be attributed to the molecular attractive forces, the molecules in the outer skin of liquid, at a liquid-air surface, experience a force directed normally towards the surface which is due to the unbalanced molecular attractions, and, in this surface layer, the intrinsic pressure of the liquid falls rapidly towards that of the atmosphere. Conversely, if it be allowed that solid molecules have usually a greater attraction for liquid molecules than these for themselves, of which there are many indications, it may be supposed that, in a liquid-solid surface skin, the internal pressure tends rapidly to approach the intrinsic pressure of the solid, and may be many times that of the free liquid. The dispersion medium in gels will exist therefore in at least two states, (1) the adsorbed liquid, *i.e.* that in the skins surrounding the solid phase which will be under an enormously increased pressure and (2) the liquid in the larger interstices of the gel held by capillary forces. Moreover, owing to the action of adsorption and, especially, of evaporation, the boundary surface of gels will be composed of a more or less tough skin which may materially affect the apparent properties of the gel. This skin is much more noticeable in some cases, and varies with the age and previous environment of the gel. Thus gelatin gels have very tough skins, agar gels, much less so, and silicic acid gels hardly noticeable skins. The cohesion of gelatin, agar and silicic acid gels varies in the same way, and since, on cutting the surface of a gelatin gel, the cohesion of the gel is enormously reduced, it seems probable that the cohesion resides largely in the surface skin. Determination of the breaking stress of 15 per cent. gelatin and 2·5 per cent. agar gels gave the small value of about 105 g. per sq. cm. for the latter, as the mean of three experiments, while the gelatin gel did not break with a load of 1282 g. per sq. cm. All these gels were about 30 hours old. It is evident therefore, that, in considering the stress-elongation curve of gels, the elasticity of the surface skin must be taken into account, and the elastic properties of this skin deserve further study.

The vapour pressures of gelatin-water mixtures have recently been studied by Gerike [1915]. Gelatin gels show a slight lowering of the vapour pressure as compared with pure water, and the concentration of the gel is of little importance below 70 per cent. gelatin. From about this composition the lowering of vapour pressure is proportional to the increase in percentage composition of gelatin: at about 94 per cent. there is a second inflexion in the vapour pressure curve. Gerike also found that the nature of the surface has a considerable influence on the behaviour of the gel in an atmosphere of water vapour. These results appear to indicate the existence of the gel water in three states, (1) free water containing solated gelatin, (2) water contained in fine capillaries, and (3) a small mass of more firmly bound water, which may correspond to that in the surface skins around the solid phase and may be called the adsorbed water.

Zsigmondy [1911] has shown that the von Nägeli hypothesis supplies the explanation of the curious hysteresis cycle in van Bemmelen's [1878, 1880] well known vapour pressure isotherms for silicic acid gels. Silicic acid gels dry to a clear transparent finely porous gel, which is practically constant in volume and only permits of volume changes within its elastic limits. The hysteresis cycle corresponds to this constant volume gel, During the progressive dehydration along the first descending branch of the curve, the gel is decreasing in volume, and the gel capillaries develop menisci at the gel surface, concave to the vapour phase, with increasing curvature corresponding to the fall in the vapour pressure. At the commencement of the hysteresis cycle the radius of curvature of the liquid menisci has reached its lowest value. The radius of curvature has now become approximately equal to the radius of the capillary. Such a meniscus has a maximum pull on the layer of liquid below, in consequence of which the gel walls are drawn together and the gel skeleton as a whole undergoes compression. The tension causes rupture of the liquid in the interior of the gel, and the appearance of the gas phase. The occurrence of innumerable gas bubbles causes the opaqueness which begins at this point. The bubbles are larger than the size of the interstices between the micellae, and envelop a number of particles of the solid phase. Finally, when the emptying of the capillaries is complete, at the lower point of the cycle, the bubbles have all coalesced and the opaqueness has disappeared. These facts indicate an extraordinarily fine structure. Larger discontinuities, of the order of Bütschli's honeycombs, must cause permanent opaqueness.

The ascent of the steeper branch during reimbibition is due to the fact that the unwetted walls develop liquid menisci with greater radii, and consequently higher vapour pressure, than before. The greater water uptake during the last part of this branch corresponds to the enlargement of the capillary volume in consequence of the diminished pressure in the gel occasioned by the gradual flattening of the menisci.

For such a gel the size of the capillaries can be calculated, and, for a reduction of vapour pressure of 6 mm., Zsigmondy finds a capillary radius of about $2{\cdot}5\,\mu\mu$.

This was completely confirmed by Anderson [1914], who replaced the imbibition water by alcohol and benzene, and determined the vapour pressure isotherms in a vacuum apparatus. The curves were of exactly the same form as for water. From the formula $r = \frac{2Ts_0}{\sigma p_0 \log p_0/p_1}$ (where r = radius of capillary, T = surface tension of imbibed liquid, s_0 = density of saturated vapour, σ = density of imbibed liquid, p_0 = ordinary vapour pressure of imbibed liquid, and p_1 = vapour pressure of liquid in capillaries) Anderson found the values:

Imbibed liquid	p_1 in cm. Hg.	r in $\mu\mu$
Water	0·84	2·74
Alcohol	2·12	5·58
Benzene	2·9	2·99

These figures indicate that the diameter of the ultimate particles composing the gel micellae is about $5\mu\mu$. Taking the thickness of the surface skin of adsorbed liquid, as calculated from van der Waal's equation, at about $0{\cdot}15\mu\mu$, and assuming a specific surface corresponding to spherical particles $5\mu\mu$ in diameter, the total volume of adsorbed liquid in a so-called 10 per cent. gelatin gel, containing 8·5 per cent. of dry gelatin, would be about 0·8 per cent. This figure agrees fairly with the value calculated from Rodewald's data below.

Organic hydrogels such as gelatin differ from silicic acid gels in the method of their formation. Whereas the gelation of the latter is irreversible, the gelation of the organic gels is reversible. The cause of this difference lies in the solubility of the organic gel substances in the dispersion medium, as has already been shown. This solubility affects the behaviour of the gel on drying. Evaporation leads to gradual deposition of solid in the pores of the micellae as these are pulled together by the increasing curvature of the liquid menisci, with the result that the gel dries to a transparent hard mass. The rehydration process must include the resolation of the deposited excess of gel substance before the full amount of water can be taken up. This is the reason why the organic gel substances will not absorb liquids in which they are insoluble. Bütschli showed, however, how to substitute such liquids for the water in a swollen gel, and Bachmann [1917] utilised his method to obtain constant volume gels of which the vapour pressure isotherms might be studied. The procedure consists in gradually hardening the gelatin, and, at the same time substituting alcohol for the water, by immersing the hydrogel, in aqueous alcohol of gradually increasing concentration. In this way a hardened gelatin alcohol-gel is obtained in which the alcohol occupies the same volume as did the water; and the alcohol can be changed for benzene, xylene and other liquids. The vapour pressure isotherms of these hardened gels were of the same type as the van Bemmelen curves for silicic acid, showing a similar hysteresis cycle corresponding to an opaque appearance in the gel. Bachmann also studied a number of other porous substances, such as hydrophane, permutite and wood charcoal, and concluded that there is a series of chemically very different substances with finely porous gel structure, showing similar vapour pressure isotherms, which correspond to the reduced vapour pressure of a liquid inclosed in amicroscopic capillaries. All the curves include a hysteresis cycle analogous to those discovered by van Bemmelen for the gels of copper oxide, iron oxide and silicic acid. The type of the hysteresis cycle is dependent neither on the nature of the imbibed liquid nor on that of the gel skeleton. Bachmann calculated that the capillaries are 30–300 times smaller than those required by Bütschli's honeycomb structure, which would have the enormous size of 700–800$\mu\mu$, and could play no part in the reduction of the vapour pressure.

From experiments on the imbibition of pure chemical compounds, Katz [1917] found that the degree of swelling is independent of the chemical nature of the swelling substance. The curves showing the relation between the degree of swelling and the vapour pressure, volume change, and heat of swelling, are similar to the corresponding curves for mixtures of water with non-volatile liquids such as sulphuric acid and glycerol. From this Katz concluded that the swelling process is to be regarded as the formation of a solid solution, and is incompatible with von Nägeli's theory. On the contrary, the question may be asked, whether the causes underlying the phenomena in both cases are not of the same nature, and whether it may not be hoped that the explanation of the similarity will be found in the sphere of action of the intra-molecular forces. This view of the imbibition process is in conformity with Proctor's experiments [1914] which show that the phenomena are dependent on the relation between the osmotic pressures and equality of products of the hydrolysable gelatin salt which is formed and of the external acid solution.

Some indication of the enormous pressures experienced by the adsorbed liquid in gels is afforded by Schroeder's experiments with gelatin [1903] and by Reinke's work with the oedometer [1879]. Working with discs of Laminaria, the latter showed that gels, separated from water by an osmotic membrane, develop pressures, in equilibrium with the water content of the gel, increasing to 41 atmospheres as the water content falls to 16 per cent. of the air-dry gel. Since air-dry Laminaria contains about 20 per cent. of water, the pressures under which the last traces of water are adsorbed must be enormous. It is impossible to ignore the apparent analogy between these pressures and the osmotic pressures of solutions, with the suggestion that both originate in the molecular attractions of the liquid and solid phases or of solvent and solute. Indeed the study of colloids abounds with indications that the difference between true and colloid solutions, and again between these and gels, is merely one of degree, and that the same general laws are applicable to all. In this connection it will be remembered that the close relation between the properties of sols and gels was pointed out by Arisz [1915] who concluded that gels should be regarded as liquids of great viscosity.

Reinke's experiment does not lead to a direct estimation of the pressure of the adsorbed liquid in gels. More insight is obtained by examination of the phenomena of swelling.

The total contraction of gel and water on imbibition by gelatin has been studied by Lüdeking [1888]. Unfortunately he used air-dry gelatin, which still contains about 15 per cent. of water, so that the volume changes observed were only a fraction of those to be expected with a dry gel. The density of the air-dry gelatin was 1·412. From the densities found by Lüdeking, the contractions in cc. have been calculated assuming the water alone to have decreased in volume.

	Specific volume	Volume in cc. of gelatin in 100 g. of gel	Contraction per cc. of water
Air dry gelatin	0·7082	—	—
10 g. gelatin + 90 g. H_2O	0·9354	7·08	0·040
25 g. gelatin + 75 g. H_2O	0·8810	17·70	0·063
50 g. gelatin + 50 g. H_2O	0·8052	35·41	0·098

These small contractions indicate that the first portions of water taken up are the more compressed. The maximum contraction corresponds to a density for the adsorbed water of about 1·11.

Information of more value is given by Rodewald's investigations on the contraction in total volume of starch and water during swelling [1897]. Rodewald used starch dried over sulphuric acid. This still contains some water, since drying *in vacuo* at 100° is necessary to drive off all the water [Dafert, 1887], but the contractions obtained should be much greater than with an air-dry gel. Assuming that the contraction is confined to the water, the volumes of 1 g. of water at each concentration have been calculated from Rodewald's results.

100 g. starch dried over H_2SO_4, containing water	Specific Volume	Vol. of adsorbed water in cc.	Vol. of 1 g. water in cc.	Vol. of 1 g. additional water, after subtracting vol. (=3·01 cc.) of first 9·8 g.
0	0·72585	—	—	—
1·17	0·72124	0·38	0·33	—
2·69	0·71319	0·78	0·29	—
5·02	0·70486	1·54	0·31	—
7·40	0·69881	2·67	0·36	—
9·70	0·69344	2·79	0·29	—
13·41	0·68727	6·60	0·49	0·91
14·95	0·68555	8·22	0·55	1·01
19·24	0·68111	9·51	0·49	0·68

It will be noted that up to about 9·8 per cent. of water, in addition to that already contained in the starch, the contraction of the adsorbed water is practically constant. Subtracting the volume of this 9·8 per cent. of water from the calculated volumes of adsorbed water at larger concentrations gives practically no contraction for the additional water, except in the last case. From these figures it is possible to get a very rough idea of the pressures under which the water is imbibed.

The work of Worthington, Richards and Matthews, and others indicates that the product of the internal pressure, P, of a liquid and its compressibility, β, is approximately constant, *i.e.* $P\beta = C$. Amagat's figures [1893] show that this is not quite true for water. But, from the anomalous nature of water as a liquid, it is probable that the relation between its internal pressure and compressibility is not quite simple. Since the available data are so meagre and have not been derived from the study of dry gels, all that can be expected is to show that the theoretical considerations lead to values of the right order.

Assuming the relation $P\beta = C$, and taking $P_0 = 1{\cdot}1 \times 10^4$, and

$$\beta_0 = 5{\cdot}2 \times 10^{-5}, \text{ gives } C = 0{\cdot}572.$$

And since

$$\beta = -\frac{1}{V}\frac{dV}{dP}$$

$$\therefore \frac{dV}{V} = -0{\cdot}572\frac{dP}{P}$$

$$\text{or } \log_{10}\frac{V_0}{V_1} = 0{\cdot}572 \log_{10}\frac{P_1}{P_0}.$$

Since the first 9·8 g. of water become 3·01 cc., the average pressure producing this contraction is given by

$$\log\frac{9{\cdot}8}{3{\cdot}01} = 0{\cdot}572 \log\frac{P_1}{1{\cdot}1 \times 10^4}$$

which gives $P_1 = 8{\cdot}6 \times 10^4$.

From the last line in the table, the additional 9·44 g. of water adsorbed become 6·50 cc., from which $P_2 = 2{\cdot}1 \times 10^4$.

The heat evolved in the contractions should be given approximately by

$$Q = \frac{(P_1 - P_0)(V_0 - V_1) \times 10^6}{42 \times 10^6} \text{ cal.}$$

$$\therefore Q_1 = 1{\cdot}2 \times 10^4 \text{ cal, and } Q_2 = 7 \times 10^2 \text{ cal}$$

which is negligible as compared with Q_1. This is the heat evolved by 100 g. of starch. The heat evolved per gram is therefore 120 cal.

Rodewald found about 30 cal. per g. for starch, which is only a quarter of this amount. There is, however, another change which takes place during swelling, in addition to the condensation of the water, and this causes absorption of heat. In accordance with Laplace's conception, the presence of a liquid will reduce the unbalanced attractions in the surface layer of the solid, in consequence of the attraction of the liquid molecules in the opposite sense. The surface tension of the solid will therefore be decreased, and expansion of the surface layers will take place, with absorption of heat. The observed heat of imbibition is, therefore, less by this amount.

The heat of imbibition of gelatin was determined by Wiedemann and Lüdeking [1885]. Here again unfortunately air-dry substance was used, so that a value of only 5·7 cal. per g. was obtained. Before the employment of only air-dry substance was realised, the low value of the heat of swelling seemed a serious difficulty in working out the present theory. A determination of the heat of swelling of gelatin dried at 110° gave 32·96 cal. per g. as the mean of two experiments, or, allowing for the heat absorbed by the solation of gelatin by the water in the calorimeter, according to Wiedemann and Lüdeking's figures, the heat of swelling becomes 33·25 cal. This value is very similar to that obtained for starch.

The so-called "thermal anomaly" of gels was discovered by von Bjerkén [1891]. Arguing from a supposititious analogy with rubber, von Bjerkén expected to find that stretched gels would likewise contract on heating. From a number of experiments which were confined to gelatin gels, of which neither the quality, composition, nor form is recorded, the expectation appeared to be substantiated. No explanation of such a phenomenon is afforded by the theory considered in this paper. However, having regard to the tough skin which forms on the surface of gelatin gels, it seemed probable that such gels would contract in one direction when heated, owing to an approach to spherical shape under the influence of thermal expansion. This contraction would be dependent on the shape of the gel, and independent of any stretching force applied to the gel except indirectly as the result of a change induced in its shape. The contraction would also be affected by the variation in the elasticity of the outer skin with age or change of environment. The single case of a non-stretched gel recorded by von Bjerkén gave a very slight expansion in the unstretched state, and only a small contraction when stretched. The definite expansion is curious, since experiments show that expansion in the direction of greatest length is due to relaxation of the skin and proceeds without limit until the gel melts. This suggests that, in the unstretched state, the gel in question had its greatest dimension in some other direction than the one under observation.

Von Bjerkén himself records a noticeable small gradual expansion, which was superposed upon the contraction effect. Doubtless this was due to the gradual relaxation of the surface skin. It was therefore determined to reinvestigate the thermal behaviour of gelatin ribbons and cylinders, and the results of over seventy experiments completely fulfil expectations. The gels showed either unlimited expansion, or contraction which varied with the age or previous history of the surface skin. Cutting or injuring the skin caused expansion instead of contraction.

No exact formula can be obtained to express the relation of the contraction to the form of the gel. An approximate one may be arrived at by supposing the volume, $V = \pi r^2 h$, of a cylinder of gel to increase, while the surface, $S = 2\pi rh + 2\pi r^2$, remains constant[1]. This gives

$$dS = 2\pi h dr + 2\pi r dh + 4\pi r dr = 0$$

or

$$dr = -\frac{r dh}{h+r}.$$

The change in volume,

$$\begin{aligned} dV &= 2\pi rh dr + \pi r^2 dh \\ &= \pi r^2 dh - \frac{2\pi r^2 h dh}{h+r} \\ &= -\frac{\pi r^2 (h-r)}{h+r} dh \end{aligned}$$

$$\therefore \frac{1}{V} \cdot \frac{dV}{d\theta} = -\frac{1}{h} \cdot \frac{h-r}{h+r} \cdot \frac{dh}{d\theta}.$$

A cylinder is, however, not so convenient to work with as a ribbon, since there is danger that thermal equilibrium may not be reached during the rapid temperature changes required to prevent the gel melting. Only a still more approximate formula for a ribbon can be deduced. By writing $\rho = \frac{\sqrt{a-b}}{2}$, where a and b are the shorter dimensions of the ribbon, the equation becomes

$$\frac{1}{V} \cdot \frac{dV}{d\theta} = -\frac{1}{h} \cdot \frac{h-\rho}{h+\rho} \cdot \frac{dh}{d\theta}.$$

Since the surface skin of agar gels is very much softer than that of gelatin gels, it would be expected that the former would show no contraction on heating. This was found to be the case. It may therefore be regarded as established that the contraction in greatest length of gelatin gels when heated is due to the constricting effect of the surface skin as the gel expands in volume in accordance with its coefficient of thermal dilation, and is independent of any stretching force applied to the gel.

In so far as the phenomena exhibited by gels depend on the interrelation between the liquid and solid phases, they should be influenced by the well known lyotrope series of dissolved salts, which affect the surface tension and the affinity of the liquid and solid phases. The connection between adsorption of solvent and swelling, and the similar effect of the Hofmann series of anions on both was pointed out by Lillie [1907]. He suggested that osmotic pressure is due to adsorption of solvent by the dissolved particles, and showed that the osmotic pressure of emulsoid sols is decreased by salts in the same order, $CNS < I < NO_3 < Br < Cl < SO_4 < CO_3$ as in the case of solutions. The diminished pressure would not appear to be due to increased aggregation of the sol particles, since the precipitating effect of the cations has not commenced at the concentrations employed, although anions hinder the precipitation in the opposite order [Pauli, 1913]. The same relations were found by Hatschek for the clearing of oil emulsions. The temperature of gelation should however be depressed in the order $CNS > I > \ldots > CO_3$ as was found by Levites [1908]. The change in the viscosity with time [Schröder, 1903] may be deduced in the same way as due to a diminution of the precipitating forces and the formation of fewer crystallisation centres, or sol particles. If the velocity of crystallisation is sufficiently reduced, gelation may be altogether prevented, as by the addition of the thiocyanate anion. The solubility of the colloid particles is temporarily increased thereby, their aggregation hindered and the sol stage prolonged. In a similar way the elasticity of gels is increased by salts which favour imbibition and *vice versa*. The double refraction of deformed sols shows the same lyotrope effect [Lieck, 1904].

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[1] Actually if the surface were inelastic the gel could not change in shape.

4. EXPERIMENTAL.

The gelatin used in all, except the earliest, of these experiments was Nelson's "Crystal" leaf gelatin. It contained 15·41 per cent. of moisture, 1·66 per cent. of ash and only a trace of phosphate. The ash appeared to be due mostly to the presence of calcium bicarbonate in the gelatin.

A quantity of this gelatin was soaked for 6 days in running distilled water and quickly evaporated until the gel corresponded to 10 per cent. of the original gelatin. This gel gave no precipitate when treated with lime-water, or sodium phosphate solution, and only a trace of precipitate in the form of finely divided bands, with silver nitrate solution. It did not give the Liesegang phenomenon until citric acid had been added. Contrary to Liesegang's experience it was then unnecessary to add additional gelatose.

The gel so prepared was, however, quite unsuitable for the investigation of the thermal anomaly of gelatin, since the gelatin had almost completely lost its coherence, together with its property of forming a hard surface skin. It was impossible to mould a cylinder or ribbon which could be handled without breaking. This behaviour was identical with that of a sample of gel similarly prepared from another specimen of gelatin. The gelatin was further purified by two precipitations by pouring the aqueous sol into alcohol.

A number of ribbons made from this gelatin had also very soft surface skins and were equally fragile. From this it appears that the cohesion of gelatin is due to a water soluble substance, probably gelatose, which is removed by soaking. Consequently the leaf gelatin was used for the experiments in its original state. The agar gels were prepared directly from ordinary commercial shredded agar.

The so-called "Thermal anomaly" of gels.

The apparatus employed by v. Bjerkén for the observation of this property consisted of an apparently thick glass beaker, provided with a stirrer, and containing paraffin oil in which the ribbon of gel was mounted. The beaker was surrounded with an outer jar containing water, by changing which the temperature of the paraffin oil bath was regulated. Since the gelatin is heated to about 40°, which is much over its melting point, it is important that the temperature changes should be as rapid as possible. Consequently a modified form of apparatus was used. This comprised a thin glass tube 10 × 2 cm., with an overflow lip above, and having a side tube 1 cm. diam. at the base, where the main tube was constricted to 5 mm. diam. Through the constricted tube a glass rod passes freely, provided with a platinum hook above for the attachment of the gelatin ribbon. The space between the rod and the tube was closed by an outer sleeve of rubber tube. The rod was long enough for the hook to be passed to the top of the main tube when fitting the ribbon. It was then carefully drawn down, until the gel was in position, and securely clamped in place. By means of the side tube a rapid stream of liquid could be passed through the main tube from either of two thermostats at the temperatures selected for the experiment. These were usually that of the room and about 40°. The leading tubes were 1 cm. in diameter and, to avoid constriction, rubber connections and pinch cocks were employed. The side tube was fitted at right angles below with a waste tube, of the same diameter, for the rapid emptying of the main tube. The fluid found most convenient was liquid paraffin B.P. Observation showed that the change in length of the gel when immersed in water is inappreciable during the short time of the experiment, and this liquid was used on some occasions as a check. Water has also the advantages of greater heat capacity and mobility. When using liquid paraffin a small correction for the difference in temperature of the bath from that of the thermostat must be applied. With water there is no correction.

The gel cylinders or ribbons were moulded with metal plates embedded in the surfaces at the two ends. The plates were provided with holes through which wires could be hooked for attachment. The lower end of the gel was hooked to the sliding glass rod, and the upper end was connected to a light lever and pointer, by means of which the position of the end of the gel could be estimated, with the aid of a lens, to within 0·001 mm. The weight of this lever caused a pull of only 6·5 g.

The preparation of the cylinders and ribbons was a matter of considerable difficulty, since it was essential to secure gels of which the surface was uninjured. Glass moulds, which were afterwards broken, were of no use. Moulds of silver foil formed round a glass tube and clamped down the side by a bull-dog clip, the edges of which had been filed square, seemed most convenient. The lower end of the tube so formed was closed by a split cork carrying the tongue of the circular plate carried above for the attachment of the gel, and was luted with paraffin wax if necessary. The upper plate was suspended by a pin through the perforation of the tongue, the cylinder filled with gelatin and allowed to set. The clamp was then removed and the cylinder weighed and slipped into a small test tube which was corked until required for use. The ribbons were moulded in troughs formed of extra stout tin foil, and kept in corked test tubes. The rate of loss of moisture from the gel was found to be fairly constant at about 0·6 per cent. in the time, about 20 hours, before measuring the expansion. From the time of taking out of the tubes it was impossible to control the loss of water. The compositions of the gels are therefore given as they were prepared, and their history recorded in each case. As a guide to their water content, the rate of loss of moisture by a sugar loaf of 10 per cent. air-dry gelatin weighing 15·147 g. exposed to the air under a raised inverted funnel was determined, see Fig. 2. To open the mould, the sheet metal was carefully peeled off the gel. Usually the surface of the gel was little damaged in the process, but it was never obtained quite perfect. Accordingly the gel was hung on a hook and painted all over with a hot gelatin sol of the same concentration. After drying for several hours in the air, the gel was carefully mounted in the apparatus and the cold liquid run through for about 30 seconds to insure temperature equilibrium. If the surface of the gel was even slightly injured in mounting, the gel subsequently gave no contraction. After 30 seconds the scale reading was taken, the cold liquid quickly run out, and a rapid current of hot liquid maintained until the maximum contraction had been recorded. This took about 15 seconds in liquid paraffin and half that time in water. The current of hot liquid was immediately changed for cold, before the gel melted, when the gel invariably expanded to beyond its original volume, the expansion continuing for some time. This appeared to be due to the softening and gradual expansion of the outer skin by the hot liquid and the pull of the light weight of the lever.

From upwards of seventy experiments, every degree of effect was obtained from zero to a more or less definite maximum contraction, as well as

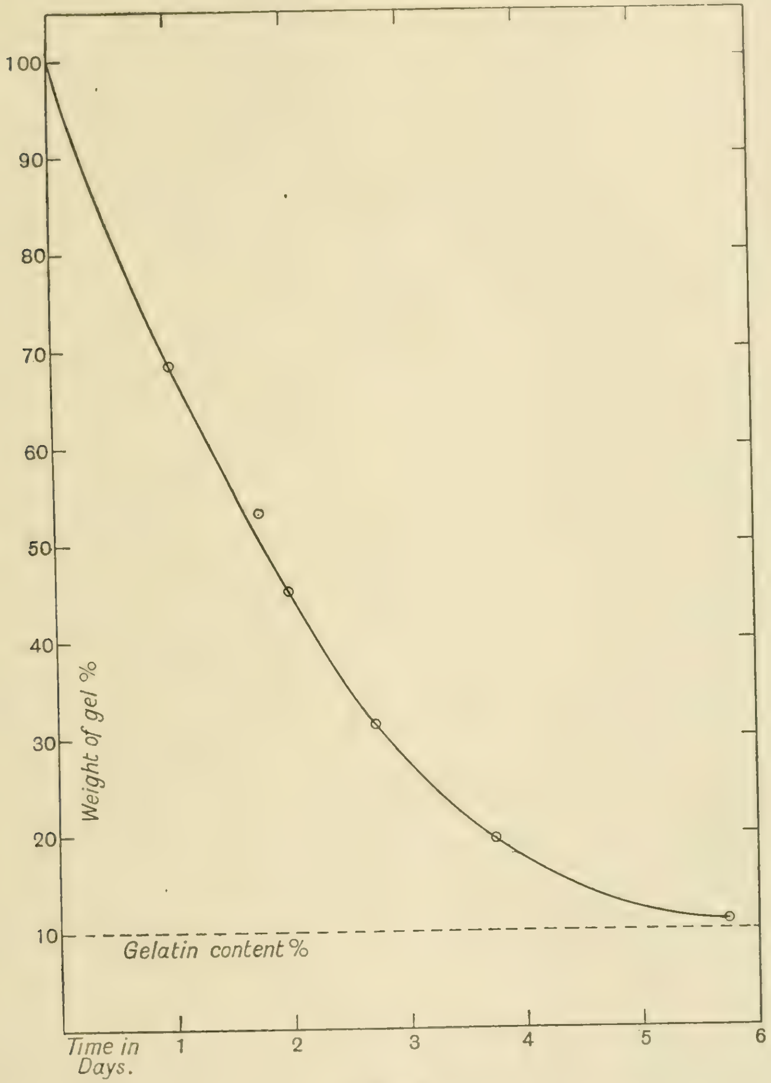

Fig. 2

indefinite expansion until melting occurred, according to the state of the outer skin of the gel. Usually very small contractions were observed on the first day, but after leaving the gel mounted in the empty apparatus over night, about 16 hrs., a much greater effect was usually obtained. Keeping the gels in oil prevented any increase in contraction with time, whether before or after the first measurement. A second heating immediately following the first gave much less, or no contraction; a third consecutive heating invariably none. Keeping for 16–24 hrs. however usually restored or increased the effect. If the heating was continued too long, the outer skin melted before the interior of the gel and rapid expansion immediately occurred. That the contraction in length was due to an actual expansion in volume was proved by the facts that cylinders of less height than diameter increased in height on heating, and that gels confined in test-tubes, so that increase of diameter was prevented, also showed a linear expansion.

On account of its lack of adhesiveness, there was no difficulty in moulding cylinders of agar gel. These were prepared of 2·5 per cent. agar. They came out of the small glass tubes without any injury, but always showed immediate expansion in length when heated.

The results of a selection of the experiments are tabulated below. The specific linear contractions are given, as well as the calculated specific volume expansions, for comparison with von Bjerkén's data. No. 5 was a cylinder with height greater than its diameter. The times are measured from the moment of painting. The dimensions are given in cm.

The results appear to indicate, either that the actual thermal expansion of fresh gelatin gels is greater than that of the contained liquid, or that it becomes so with time. A number of experiments by various methods were made to determine this effect. Unexpected difficulties were encountered in obtaining concordant results, and when such had been obtained by one method, the values were found to differ greatly from an equally concordant set resulting from a different process. The question requires further study, and it is proposed to investigate the densities and coefficients of expansion of pure dry gelatin and agar and of a series of gels and sols of different concentration and age. This will also determine the total contraction of gel and water on swelling.

The Heat of Imbibition of Gelatin.

The heat of imbibition of air-dry sheet gelatin was found by Wiedemann and Lüdeking [1885] from three experiments as 6·2, 5·3 and 5·6 cal. giving a mean value of 5·7. The same observers obtained the value – 3·7 as the heat of solation of 1 g. of gelatin.

The gelatin for the present experiment was dried in the air oven at 110°

No.	Age	Concentration, etc.	Dimensions in cm.	Liquid in bath	$\frac{dL}{d\theta}$	$\frac{1}{h}\frac{dL}{d\theta}$	$\frac{1}{V}\cdot\frac{dV}{d\theta} = -\frac{1}{h}\frac{h-r}{h+r}\frac{dh}{d\theta}$
3	24 hrs.	10 % gelatin, old sample	7·5 × 1·3	water	$-0{\cdot}0030$	$-0{\cdot}0_3 4$	$+0{\cdot}0_3 3$
4	24 ,, ...	,, ,, ,,	7·5 × 1·3	,,	$-0{\cdot}0030$	$-0{\cdot}0_3 4$	$+0{\cdot}0_3 3$
5	3½ ,, ...	,, ,, ,,	1·0 × 1·25	,,	Expanded $+0{\cdot}0_3 138$	$+0{\cdot}0_3 138$	$+0{\cdot}0_4 28$
13	3 ,, ...	,, ,, ,,	5·9 × 0·93	,,	$-0{\cdot}0_3 188$	$-0{\cdot}0_3 32$	$+0{\cdot}0_3 2$
16	Reheated immediately	Same gel	—	,,	No contr.	—	—
17	16 hrs.	10 % Crystal gelatin ...	4·5 × 0·75	liquid paraffin	$-0{\cdot}0_3 67$	$-0{\cdot}0_3 15$	$+0{\cdot}0_3 13$
18	Reheated	Same gel	—	,,	$-0{\cdot}0_3 49$	$-0{\cdot}0_3 11$	—
19	16 hrs.	10 % Crystal gelatin ...	3·9 × 0·65	,,	$-0{\cdot}0_3 33$	$-0{\cdot}0_4 8$	$+0{\cdot}0_4 7$
20	After another 24 hrs. in app.	Same gel lengthened by hanging	4·1 × 0·55	,,	$-0{\cdot}0_2 179$	$-0{\cdot}0_3 437$	$+0{\cdot}0_3 381$
22	16 hrs.	10 % Crystal gelatin ...	4·3 × 0·6	,,	Expanded	—	—
23	24 hrs. longer in app.	Same gel	4·5 × 0·55	,,	$-0{\cdot}0_2 296$	$-0{\cdot}0_3 658$	$+0{\cdot}0_3 578$
27	16 hrs. hung over water in covered beaker	10 % Crystal gelatin ...	6·3 × 1·1 × 0·3	,,	$-0{\cdot}0_3 22$	—	—
28	3½ hrs. later ...	Same gel	6·7 × 1·0 × 2·5	,,	$-0{\cdot}0_2 249$	$-0{\cdot}0_3 372$	$+0{\cdot}0_3 291$
29	16 hrs.	10 % Crystal gelatin, surface injured	4·6 × 0·66	,,	Expanded	—	—
30	21 hrs. longer in app.	Same gel	Unchanged	,,	$-0{\cdot}0_2 198$	$-0{\cdot}0_3 430$	$+0{\cdot}0_3 386$
31	3 hrs.	10 % Crystal gelatin ...	5·9 × 1·2 × 0·3	,,	$-0{\cdot}0_3 93$	$-0{\cdot}0_3 157$	$+0{\cdot}0_3 144$
32	3 ,, ...	,, ,, ,,	5·1 × 1·2 × 0·33	,,	Expanded	—	—
33	3 ,, ...	,, ,, ,,	5·0 × 1·1 × 0·3	,,	$-0{\cdot}0_2 111$	$-0{\cdot}0_3 222$	$+0{\cdot}0_3 192$
34	Same gel 24 hrs. longer in app.	,, ,, ,,	5·3 × 1·0 × 0·26	,,	$-0{\cdot}0_2 439$	$-0{\cdot}0_3 809$	$+0{\cdot}0_3 736$
36	24 hrs. in app. ...	,, ,, ,,	6·1 × 1·1 × 0·35	,,	$-0{\cdot}0_2 108$	$-0{\cdot}0_3 178$	$+0{\cdot}0_3 161$
54	7 hrs.	12·5 % Crystal gelatin ...	5·3 × 1·25 × 0·35	,,	$-0{\cdot}0_3 26$	$-0{\cdot}0_4 49$	—
56	7 ,, ...	,, ,, ,,	5·1 × 1·1 × 0·3	,,	Expanded	—	—
57	Same gel 24 hrs. longer in app.	,, ,, ,,	5·2 × 1·1 × 0·3	,,	$-0{\cdot}0_2 498$	$-0{\cdot}0_3 958$	$+0{\cdot}0_3 731$
58	24 hrs.	,, ,, ,,	4·9 × 1·0 × 0·3	,,	$-0{\cdot}0_2 233$	$-0{\cdot}0_3 476$	$+0{\cdot}0_3 364$
68	5 ,, ...	15 % Crystal gelatin ...	5·7 × 1·3 × 0·2	water	$-0{\cdot}0_2 242$	$-0{\cdot}0_3 428$	$+0{\cdot}0_3 326$
69	5 ,, ...	,, ,, ,,	5·9 × 1·3 × 0·3	,,	$-0{\cdot}0_2 223$	$-0{\cdot}0_3 379$	$+0{\cdot}0_3 288$
70	Same gel 24 hrs. longer in app.	,, ,, ,,	5·9 × 1·1 × 0·3	,,	$-0{\cdot}0_2 668$	$-0{\cdot}0_2 1132$	$+0{\cdot}0_3 918$

On account of the approximate nature of the formulae and of the magnitude of the effect of small errors in measurement and of irregularities in form, on the calculated volumes of the gels, the figures are not of great intrinsic value.

for about 24 hours until constant in weight. The loss corresponded to 15·70 per cent. of moisture.

A small Dewar vacuum flask, holding about 150 cc., was used as calorimeter and was provided with a mechanical glass stirrer so that the rate of cooling was nearly constant. The temperatures were indicated by a Beckmann thermometer. The change of temperature by radiation per degree difference was only 0·0015° per 15 seconds. Practically the whole of the heat was evolved within about four minutes. The results were corrected by Regnault's method. Two determinations gave:

	(1)	(2)
Gelatin taken	3·942	2·083
Total water equivalent	147·841	139·845
Initial temperature of gelatin	21·2°	19·2°
Initial temperature of calorimeter	20·500°	18·486°
Final temperature	21·413°	18·977°
Rise in temperature	0·913°	0·491°
Corresponding heat value	132·368 cal.	68·664 cal.
Heat added with gelatin	1·242 ,,	0·656 ,,
Heat of swelling per g. of dry gelatin ...	33·264 ,,	32·649 ,,
Heat absorbed by solation of gelatin per g....	0·177 ,,	0·304 ,,
Corrected heat of imbibition	33·541 ,,	32·953 ,,
Mean	—	33·25 ,,

The actual heat of swelling is greater than the observed value, in consequence of the absorption of heat by the solation of some of the gelatin by the water in the calorimeter. This is proportional to the mass of water used. The exact amount solated during the time of the experiment is however unknown. An experiment showed that 100 g. of water are capable of taking up 0·114 g. of solid matter from sheet gelatin at 20·2° during the time of the experiment, which corresponds to 0·135 g. of air-dry gelatin. Using this value and Wiedemann and Lüdeking's figure for the heat of solation, the quantities of heat absorbed are 0·177 cal. and 0·304 cal. per gram respectively.

The values obtained do not agree very well. This may be due to the great influence of the previous thermal history of the gelatin upon its structure. It will be noticed that Wiedemann and Lüdeking's results showed a similar variation.

The writer wishes to express his indebtedness to Prof. J. C. Philip for the kind loan of the calorimetric apparatus.

SUMMARY.

The low diffusion constant of the natural emulsoids, by retarding the crystallisation of the hot sols, allows the accumulation of a large excess concentration, which, together with their large value of K in von Weimarn's formula, causes gelation on cooling. The small crystallisation velocity, due to this low diffusion constant, is also responsible for the permanency of the gels and the hysteresis of the sols, since it prevents subsequent development of larger crystals in gels and retards changes in the aggregation of sol particles.

The viscosity changes in the sols of the natural emulsoids, by heating, are seen to be in conformity with Einstein's formula, since thermal changes cause alterations in the numbers, as well as the size, of the crystallisation centres.

The application of von Weimarn's theory to the reversible sol-gel transformation implies that gels should comprise two phases: an ultramicroscopic solid phase, bathed in a liquid dispersion medium, from which it has crystallised, and which it retains partly by molecular and partly by capillary forces.

Consideration of the conditions of occurrence of spherites shows that complex organic substances and such as are highly aggregated in solution tend to crystallise in this form, and this leads to the supposition that the ultimate solid particles of gels are spherites. This is in conformity with the ultramicroscopic appearance, and receives further support from the fact that, by suitable means, gelatin can be made to assume the form of microscopic spheres.

The specific surface of gels is very large, and consequently the volume of liquid adsorbed at the liquid-solid boundary surfaces is appreciable. This liquid is under an internal pressure intermediate between those of the solid and of the liquid. This intermediate value of the internal pressure is much greater than that of the free liquid and conditions certain peculiarities in the properties of gels.

The theory forms a basis for the coordination of the properties of gels, and those predicted for such disperse systems are in accordance with the phenomena exhibited by gels. The experimental data at present available are insufficient for a complete mathematical discussion of the theory. Where the previous records are at variance with the theory, the data have been found to be in error.

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XXX. THE EFFECT OF ACIDS ON THE GROWTH OF *BACILLUS COLI.*

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The original proposition advanced by Michaelis and Marcora [1912] stated that the final acid reaction of *B. coli* constituted a "physiological constant" of the organism, since cultures in lactose bouillon ceased activity at a hydrogen ion concentration $[H^\cdot]$ of $1 \times 10^{-5} N$. The results of their experiments were soon challenged by Brünn [1913] who showed that an acid reaction of $P_H = 5 \cdot 0$ permitted, but that $P_H = 4 \cdot 7$ inhibited growth of *B. coli*.

More recently Clark and Lubs [1915, 1, 2 and 3] have examined the conditions affecting the growth of *B. coli* of the low-gas-rated group, and they find a certain connection between the type of colon bacillus used, and the final acid reaction it produces.

Clark [1915] determined the final $[H^\cdot]$ attained by cultures of *B. coli* and although he agrees with the hypothesis of Michaelis and Marcora, he presents the following additional facts:

(*a*) In a medium containing 1 per cent. Witte peptone and 1 per cent. dextrose the final acid reactions attained are:

'Human' Coli $P_H = 4 \cdot 82$
'Bovine' ,, $P_H = 4 \cdot 7 – 4 \cdot 8$
'Grain' ,, $P_H = 5 \cdot 01$.

He suggests that these reactions may be 'specific' for the respective varieties of *B. coli*, and that they may be of value for diagnostic purposes.

(*b*) A higher degree of acidity is produced in dextrose than in lactose media; *e.g.*, the same strain of *B. coli* produced in 1 per cent. Witte peptone + 1 per cent. dextrose a final reaction of $P_H = 4 \cdot 26$ while in 1 per cent. Witte peptone + 1 per cent. lactose only $P_H = 4 \cdot 56$ was attained.

(*c*) In the more highly buffered media lower H˙ concentrations are produced.

In one series of his experiments a very significant result is obtained, viz. that the addition of various amounts of sterile lactic acid to the Witte peptone-dextrose medium before inoculation causes considerable variations of the final acid reaction attained by the cultures of *B. coli* in the acidified media.

These facts seem to cast considerable doubt upon the validity of the 'physiological constant' hypothesis, and the recent researches of Wolf and Harris [1917] on the behaviour of *B. sporogenes* and *B. perfringens* in acid media indicate the desirability of further investigation of the final [H˙] reached by cultures of *B. coli*.

Again, Cole and Onslow [1915] claim to be able to differentiate the organisms of the *B. typhosus* group by the velocity with which they attain a certain degree of acidity. Wolf and Harris [1917] state, however, that in the case of *B. sporogenes* and *B. perfringens* this velocity is affected by the initial [H˙] of the medium.

It was thought desirable, therefore, to undertake further examination of the behaviour of *B. coli* in different acid media. Several important questions at once suggest themselves, *e.g.*

(*a*) Do various strains of *B. coli* behave similarly when subjected to similar conditions?

(*b*) Does the nature and degree of acidity of the original medium affect the final reaction of the culture of *B. coli* grown in it?

(*c*) What is the mechanism of the action of 'Grain' Coli?

The present paper embodies results obtained in working on questions (*a*) and (*b*), and it is hoped to present additional results, together with a consideration of (*c*) in a later paper.

The main points now investigated are:

(i) Do different strains of the same variety of *B. coli* (*e.g.* human or bovine) react in the same way when exposed to similar conditions?

(ii) Are there any differences between the behaviour of 'human' and 'bovine' coli, such as may be made use of for diagnostic purposes?

(iii) Is the same strain of *B. coli* differently affected by different media?

I. MATERIAL AND EXPERIMENTAL METHODS.

Material.

The strains of *B. coli* used in these experiments were prepared as follows. Smears of human and bovine faeces were inoculated into a medium containing 2 per cent. glucose, 2 per cent. peptone water; and at the end of 24 hours MacConkey broth was inoculated from the growths so obtained. At the end of a further 24 hours the culture produced in the MacConkey broth was used for the inoculation of fresh MacConkey broth and from this, at the end of a further 24 hours, inoculation of agar slopes was effected. The strains thus produced were subjected to microscopic examination, single colonies were transferred to new agar slopes and from the daughter colonies developed thereon the pure strains used in the experiments were obtained. In addition to a large number of strains of human and bovine *B. coli* thus prepared a pure strain of *B. coli*, referred to in the paper as *B. coli oxf.* was obtained from Dr A. D. Gardner of the Oxford University Pathological (Standards) Laboratory, and this was used in the preliminary experiment for comparison of the different strains of human coli.

The strains of *B. coli* specially prepared for the experiments were lettered H_1, H_2, H_3 etc. (human) and B_1, B_2, B_3 etc. (bovine).

Media.

Two media were used:

(*a*) 2 per cent. glucose + 2 per cent. peptone water, prepared by the tryptic digestion of caseinogen (Lait Proto A) as described by Cole and Onslow, from which the tubes of acidified media were prepared by a method similar to that of Wolf and Harris.

(*b*) Glucose-phthalate mixture.

This was prepared as follows:

8·8 g. of anhydrous disodium monohydrogen phosphate
2·0 g. of potassium acid phthalate (recrystallised)
1·0 g. of aspartic acid
4·0 g. glucose

were dissolved in distilled water, the volume of the solution being made up to 800 cc.

This solution was then brought to the required concentration by taking, for the preparation of each 100 cc. of acid medium, 50 cc. of phthalate solution; x cc. of $N/5$ acid and $(50 - x)$ cc. of distilled water.

This glucose-phthalate medium was selected for use in these experiments because it had been found by Clark and Lubs to be a medium in which

B. coli can be grown with considerable facility, and it was also known to have a considerable 'buffer' effect: this being more marked than is the case with the glucose-peptone medium. *It was found that the gas production due to the activity of* B. coli *was much less in glucose phthalate than in glucose peptone.*

Experimental Methods.

For each series of experiments A, B, C, D, etc., a number of sets of tubes, 1, 2, 3, 4, etc., were prepared, each tube containing 10 cc. of a solution containing medium, acid, and water. The set of tubes containing the least amount of acid was numbered 1, the next more acid set 2,—the most acid set of tubes in each series receiving the highest number. In general, each set consisted of eight tubes of the same mixture, and each series comprised eight or ten sets of tubes. Into each tube of medium a sterilised Durham tube, for the collection of evolved gas, was introduced. The tubed media were sterilised at 100° for one hour per diem on each of three successive days. One tube of each set was used for the determination of the initial reaction of the sterilised medium. The reaction was determined at about 20°, and corrected to 20° when necessary. The H-electrode was used, and the P_H of the tubed medium was calculated from the E.M.F. so determined. The values of P_H were used in constructing the titration curves for the known media and acids employed.

The amounts of acid added to the media were so regulated as to produce a number of mixtures having P_H ranging from 4·0 to 7·0.

For each acid and medium a titration curve was constructed, in which the number of cc. of $N/5$ acid present in 10 cc. of the solution was plotted against the absolute acid reaction (P_H) produced. These curves showed that in order to produce a given acid reaction (P_H) in a medium, it was necessary to add to a given volume of it, a smaller mass of a highly dissociated acid (*e.g.* hydrochloric acid) than of a less dissociated acid (*e.g.* acetic acid).

The addition of the less dissociated acids soon produces an acid reaction (P_H) at which subsequent considerable additions of acid result in little change of reaction. In the more highly buffered media this point is more quickly attained. There does not appear to be any marked 'specificity' of action due to the *anion* groups of the various acids upon the media. Titration curves showing the effect of different acids upon 2 per cent. glucose peptone have been given by Wolf and Harris and as similar curves were obtained in the present experiments it was thought unnecessary to reproduce them. As the curve for glucose phthalate is not known it is shown in Fig. 1.

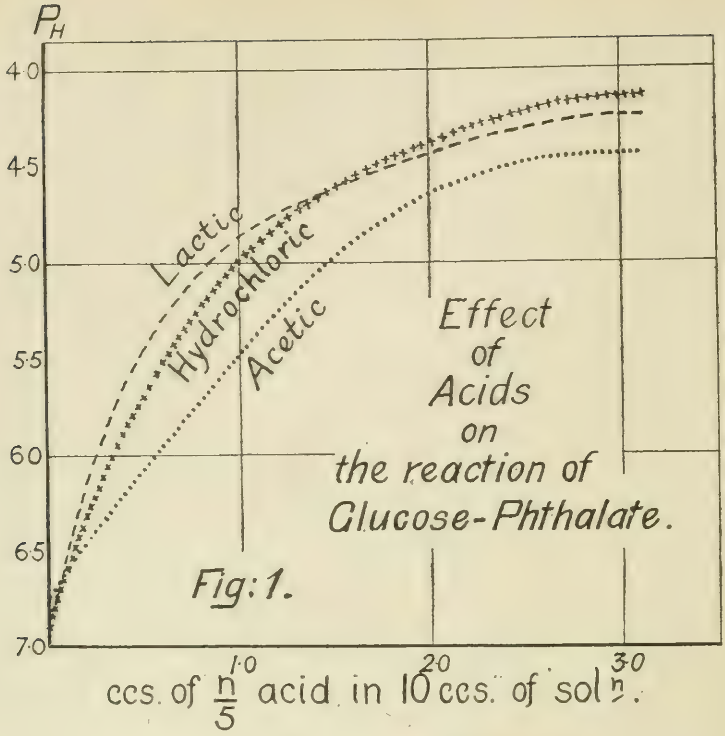


Fig: 1.

Results of inoculation experiments.

In the data given below strains H_1, H_2, etc. are *B. coli* obtained from human faeces: B_1, B_2, etc. prepared from bovine faeces, and "final P_H" is the lowest value recorded during a period (unless otherwise stated) of 168 hours.

+ indicates that fermentation occurred in the tube so marked; – means failure to ferment.

Table I. *Medium, 2 per cent. glucose peptone. Acid, hydrochloric.*

Series Z_1				*Series Z_2*			
Inoculated with H_4 18 hrs. old				Inoculated with B_4 18 hrs. old			
Tube	Result	Initial P_H reaction	Final P_H reaction	Tube	Result	Initial P_H reaction	Final P_H reaction
1	+	6·66	4·65	1	+	6·66	4·64
2	+	6·23	4·54	2	+	6·23	4·50
3	+	5·73	4·45	3	+	5·73	4·46
4	+	5·42	4·37	4	+	5·42	4·43
5	+	4·95	4·33	5	+	4·95	4·42
6	+	4·79	4·30	6	+	4·79	4·35
7	+	4·64	4·29 –	7	+	4·64	4·30 –
8	+	4·45	4·29 –	8	+	4·45	4·31

(In both of these series all the tubes fermented, the most acid reactions obtained being: Series Z_1, Tubes 7 and 8 $P_H = 4·29$, and Series Z_2, Tube 7 $P_H = 4·30$.)

	Series Y_1			*Series Y_2*	
	Inoculated with H_1 18 hrs. old			Inoculated with B_2 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	5·89	+	4·50	+	4·53
2	5·24	+	4·40	+	4·41
3	4·91	+	4·38	+	4·39
4	4·62	+	4·30	+	4·30
5	4·02	–	—	–	—
6	3·96	–	—	–	—
7	3·53	–	—	–	—

The tubes of Series Y_1 and Y_2 were examined 72 hours after inoculation.

The most acid tubes to ferment were those numbered 4 in each series, both of which had an initial reaction of $P_H = 4·62$ and reached a final reaction of $P_H = 4·30$. The next tube, No. 5 in each series, with initial reaction of $P_H = 4·02$ failed to ferment.

	Series X_1			*Series X_2*	
	Inoculated with H_1 16 hrs. old			Inoculated with B_2 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	5·87	+	4·47	+	4·50
2	5·27	+	4·35	+	4·34
3	4·65	+	4·30	+	4·295
4	4·34	–	—	–	—
5	4·16	–	—	–	—
6	4·03	–	—	–	—
7	3·77	–	—	–	—
8	3·70	–	—	–	—

The tubes were examined 90 hours after inoculation.

In series X_1 the most acid tube to ferment was No. 3 with reaction $P_H = 4·65$, the acidity of which rose to $P_H = 4·30$. In series X_2 a similar result was obtained and a final value $P_H = 4·295$ was reached. It is noteworthy that in both series Tube No. 4 with an initial $P_H = 4·34$ failed to ferment.

Series O

Inoculated with *B. coli oxf.* 16 hrs. old

Tube	Initial P_H reaction	Result	Final P_H reaction
1	6·63	+	4·79
2	6·06	+	4·70
3	5·80	+	4·66
4	5·18	+	4·54
5	4·93	+	4·50
6	4·62	+	4·30
7	4·34	–	—
8	4·21	–	—

The most acid tube to ferment was No. 6 with initial reaction of $P_H = 4{\cdot}62$ and final reaction of $P_H = 4{\cdot}30$. It must be noted, however, that the next tube, No. 7, which failed to ferment had an initial reaction of $P_H = 4{\cdot}34$ which is less acid than the final value reached by the fermented No. 6.

The existence of this phenomenon in the case of *B. sporogenes* and *B. perfringens* has already been recorded by Wolf and Harris.

	Series S_1			*Series S_2*	
	Inoculated with H_8 18 hrs. old			Inoculated with B_8 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	7·14	+	4·53	+	4·53
2	5·17	+	4·39	+	4·45
3	4·52	+	4·30	+	4·34
4	4·29	+	4·26?	–	—
5	4·00	–	—	–	—
6	3·695	–	—	–	—
7	3·525	–	—	–	—
8	3·37	–	—	–	—

In Series S_1 the most acid tube certainly to ferment was No. 3, with initial $P_H = 4{\cdot}52$ which rose to $P_H = 4{\cdot}30$. Tube No. 4 with initial acid reaction $P_H = 4{\cdot}29$ rose to $P_H = 4{\cdot}26$ although the usual signs of fermentation were absent. Tube No. 5 with $P_H = 4{\cdot}00$ failed to ferment.

In Series S_2 the most acid tube to ferment was No. 3 with an initial acid reaction $P_H = 4{\cdot}52$ which rose to $P_H = 4{\cdot}34$. Tube No. 4 with $P_H = 4{\cdot}29$ failed to ferment.

TABLE II. *Medium, 2 per cent. glucose peptone. Acid, lactic.*

	Series W_1			*Series W_2*	
	Inoculated with H_3 16 hrs. old			Inoculated with B_4 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·46	+	4·60	+	4·61
2	5·46	+	4·48	+	4·47
3	4·53	+	4·47	+	4·46
4	4·39	–	—	–	—
5	4·15	–	—	–	—
6	3·98	–	—	–	—
7	3·83	–	—	–	—
8	3·73	–	—	–	—

In Series W_1 the most acid tube to ferment was No. 3 with acid reaction $P_H = 4{\cdot}53$ which rose to $P_H = 4{\cdot}47$. The next tube, No. 4, with $P_H = 4{\cdot}39$ failed to ferment.

In Series W_2 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4·53$, which rose to $P_H = 4·46$. The next tube, No. 4, with $P_H = 4·39$ failed to ferment.

		Series T_1		*Series T_2*	
	Inoculated with H_8 18 hrs. old			Inoculated with B_8 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·90	+	4·69	+	4·70
2	4·88	+	4·61	+	4·60
3	4·62	+	4·57	+	4·56
4	4·44	–	—	–	—
5	4·275	–	—	–	—
6	4·08	–	—	–	—
7	3·93	–	—	–	—
8	3·70	–	—	–	—

In Series T_1 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4·62$ which rose to $P_H = 4·57$. The next tube, No. 4, with $P_H = 4·44$ failed to ferment.

In Series T_2 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4·62$ which rose to $P_H = 4·56$. The next tube, No. 4, with $P_H = 4·44$ failed to ferment.

TABLE III. *Medium, 2 per cent. glucose peptone. Acid, acetic.*

		Series V_1		*Series V_2*	
	Inoculated with H_8 16 hrs. old			Inoculated with B_8 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	7·14	+	4·52	+	4·54
2	5·39	+	4·60	+	4·61
3	4·83	+	4·77	+	4·79
4	4·60	–	—	–	—
5	4·46	–	—	–	—
6	4·41	–	—	–	—
7	4·35	–	—	–	—
8	4·28	–	—	–	—

In Series V_1 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4·83$ which rose to $P_H = 4·77$. The next tube, No. 4, with $P_H = 4·60$ failed to ferment except in one doubtful case in which the P_H rose to 4·58.

In Series V_2 the most acid tube to ferment was No. 3, with acid reaction $P_H = 4{\cdot}83$ which rose to $P_H = 4{\cdot}79$. The next tube, No. 4, with $P_H = 4{\cdot}60$ failed to ferment.

TABLE IV. *Medium, glucose phthalate. Acid, hydrochloric.*

		Series A_1		*Series A_2*	
	Inoculated with H_4 18 hrs. old			Inoculated with B_5 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·90	+	5·73	+	5·76
2	6·71	+	5·35	+	5·38
3	6·45	+	5·06	+	5·06
4	6·25	+	4·93	+	4·94
5	5·87	+	4·83	+	4·83
6	5·66	+	4·77	+	4·76
7	5·10	+	4·72	+	4·71
8	4·40	–	—	–	—

In Series A_1 the most acid tube to ferment was No. 7 with acid reaction $P_H = 5{\cdot}10$ which rose to $P_H = 4{\cdot}72$. The next tube, No. 8, with $P_H = 4{\cdot}40$ failed to ferment.

In Series A_2 the most acid tube to ferment was No. 7 with acid reaction $P_H = 5{\cdot}10$ which rose to $P_H = 4{\cdot}71$. The next tube, No. 8, with $P_H = 4{\cdot}40$ failed to ferment.

Series N

Inoculated with *B. coli oxf.* 16 hrs. old

Tube	Initial P_H reaction	Result	Final P_H reaction
1	6·69	+	5·21
2	6·56	+	5·20
3	6·44	+	5·18
4	6·15	+	5·12
5	5·89	+	5·05
6	5·70	+	4·94
7	5·37	+	4·91
8	4·85	+	4·78
9	4·31	–	—
10	3·70	–	—
11	3·31	–	—

The most acid tube to ferment was No. 8, with acid reaction $P_H = 4{\cdot}85$ which rose to $P_H = 4{\cdot}78$. The next tube, No. 9, with $P_H = 4{\cdot}31$ failed to ferment.

	Series C_1			Series C_2	
	Inoculated with H_4 18 hrs. old			Inoculated with B_4 18 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	7·11	+	5·60	+	5·43
2	6·80	+	5·11?	+	4·99?
3	6·69	+	4·99?	+	4·96?
4	6·52	+	5·05	+	5·04
5	6·27	+	5·01	+	5·01
6	6·13	+	4·93	+	4·96
7	5·95	+	4·94	+	4·88
8	5·77	+	4·89	+	4·91
9	5·01	+	4·75	+	4·76
10	4·96	+	4·68	+	4·75
11	4·41	–	—	–	—

In Series C_1 the most acid tube to ferment was No. 10, with acid reaction P_H = 4·96 which rose to 4·68. The next tube, No. 11, with P_H = 4·41 failed to ferment.

In Series C_2 the most acid tube to ferment was No. 10, with initial reaction P_H = 4·96 which rose to 4·75. The next tube, No. 11, with P_H = 4·41 failed to ferment.

TABLE V. *Medium, glucose phthalate. Acid, lactic.*

	Series K_1			Series K_2	
	Inoculated with H_7 16 hrs. old			Inoculated with B_7 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·90	+	5·75	+	5·75
2	6·00	+	5·02	+	5·05
3	5·55	+	4·94	+	4·95
4	5·20	+	4·82	+	4·85
5	5·10	+	4·78	+	4·76
6	4·99	+	4·78	+	4·79
7	4·85	+	4·76	+	4·75
8	4·71	–	—	–	—
9	4·55	–	—	–	—
10	4·44	–	—	–	—
11	4·26	–	—	–	—

In Series K_1 the most acid tube to ferment was No. 7, with an acid reaction of P_H = 4·85 which rose to P_H = 4·76. The next tube, No. 8, with P_H = 4·71 did not ferment.

In Series K_2 the most acid tube to ferment was No. 7, with an acid reaction of P_H = 4·85 which rose to P_H = 4·75. The next tube, No. 8, with P_H = 4·71 failed to ferment.

TABLE VI. *Medium, glucose phthalate. Acid, acetic.*

	Series D_1			*Series D_2*	
	Inoculated with H_8 16 hrs. old			Inoculated with B_8 16 hrs. old	
Tube	Initial P_H reaction	Result	Final P_H reaction	Result	Final P_H reaction
1	6·85	+	5·13	+	
2	6·06	+	5·16	+	5·18
3	5·56	+	5·20	+	5·25
4	4·98	+	4·93	+	4·90
5	4·78	–	—	–	—
6	4·60	–	—	–	—
7	4·50	–	—	–	—

In Series D_1 the most acid tube to ferment was No. 4, with initial acid reaction $P_H = 4·98$ which rose to 4·93. In one tube of No. 5 (initial $P_H = 4·78$) the acid reaction rose to $P_H = 4·74$ but other signs of fermentation were absent. The next tube, No. 6, with $P_H = 4·60$ failed to ferment.

In Series D_2 the most acid tube to ferment was No. 4, with acid reaction $P_H = 4·98$ which rose to $P_H = 4·90$. The next tube, No. 5, with $P_H = 4·78$ failed to ferment.

TABLE VII. *Values of final acid reaction* (P_H) *of different strains of* B. coli *in different media and in the presence of different acids.*

		"Human" Coli			"Bovine" Coli		
Medium	Acid	Series	Strain	Final P_H	Series	Strain	Final P_H
2 % glucose peptone	Hydrochloric	O	Oxf.	4·30	—	—	—
		Z_1	H_4	4·29	Z_2	B_4	4·30
		Y_1	H_1	4·30	Y_2	B_2	4·30
		X_1	H_1	4·30	X_2	B_2	4·29
		S_1	H_8	4·30 (4·26?)	S_2	B_8	4·34
		Average value P_H=4·30			Average value P_H=4·31		
2 % glucose peptone	Lactic ...	W_1	H_3	4·47	W_2	B_4	4·46
		T_1	H_8	4·57	T_2	B_8	4·56
		Average value P_H=4·52			Average value P_H=4·51		
2 % glucose peptone	Acetic ...	V_1	H_8	4·77	V_2	B_8	4·79
Glucose phthalate	Hydrochloric	A_1	H_4	4·72	A_2	B_5	4·71
		A_3	H_4	4·72	—	—	—
		N	Oxf.	4·78	—	—	—
		C_1	H_4	4·68	C_2	B_4	4·75
		Average value P_H=4·71			Average value P_H=4·73		
Glucose phthalate	Lactic ...	K_1	H_7	4·76	K_2	B_7	4·75
Glucose phthalate	Acetic ...	D_1	H_8	4·88 (4·74?)	D_2	B_8	4·90 (4·82?)

Reaction resultants.

From the values shown in Tables I to VI curves giving the reaction resultants of the various strains of *B. coli* in 2 per cent. glucose peptone, and the glucose phthalate mixture were drawn by the method of Wolf and Harris [1917]. These show the behaviour of the organisms in these media in the presence of hydrochloric, lactic, and acetic acids, the initial acid reactions (P_H) being plotted against those finally produced (Figs. 2 ff.).

CONSIDERATION OF EXPERIMENTAL DATA.

It is now possible to suggest some conclusions relative to the questions originally proposed for investigation.

1. A comparison of the values of the final acid reactions shown in Tables I to VI indicates no specificity of reaction of the different strains of *B. coli*. This may be illustrated by the following examples:

(*a*) H_4 caused the acid reaction of glucose peptone and hydrochloric acid to rise from $P_H = 4{\cdot}64$ to 4·29, while H_1 caused a rise of P_H from 4·61 to 4·30, and *Oxf.* produced a rise of P_H from 4·62 to 4·30.

When glucose phthalate was the medium used, the results support the same conclusion, for example, *Oxf.* caused the reaction of the medium to rise from $P_H = 5{\cdot}95$ to 4·94, while H_4 caused a rise from $P_H = 5{\cdot}87$ to 4·83.

It may be concluded, therefore, that the different strains of the same variety of *B. coli* react in the same way when exposed to similar conditions.

2. In no single case during the whole series of experiments, did any appreciable difference develop between the behaviour of "human" and "bovine" strains of *B. coli* (cf. Table VII).

It may be concluded therefore that the existence of differences between the behaviour of "human" and "bovine" coli such as may be of diagnostic value has not been demonstrated. In many of the experiments the two types of *B. coli* examined although fermenting under identical conditions behave identically at every stage of the experiment.

3. The experimental data lead to the conclusion that the same strain of *B. coli* is differently affected when allowed to ferment in different media. This is true both as to the final acid reaction attained in the presence of a given acid, and as to the rapidity with which this final reaction is reached. In Table I, Series Z_1, and Table IV, Series A_1, may be seen the results obtained by the fermentation of strain H_4 in glucose peptone and hydrochloric acid, and glucose phthalate and hydrochloric acid, respectively. When media to

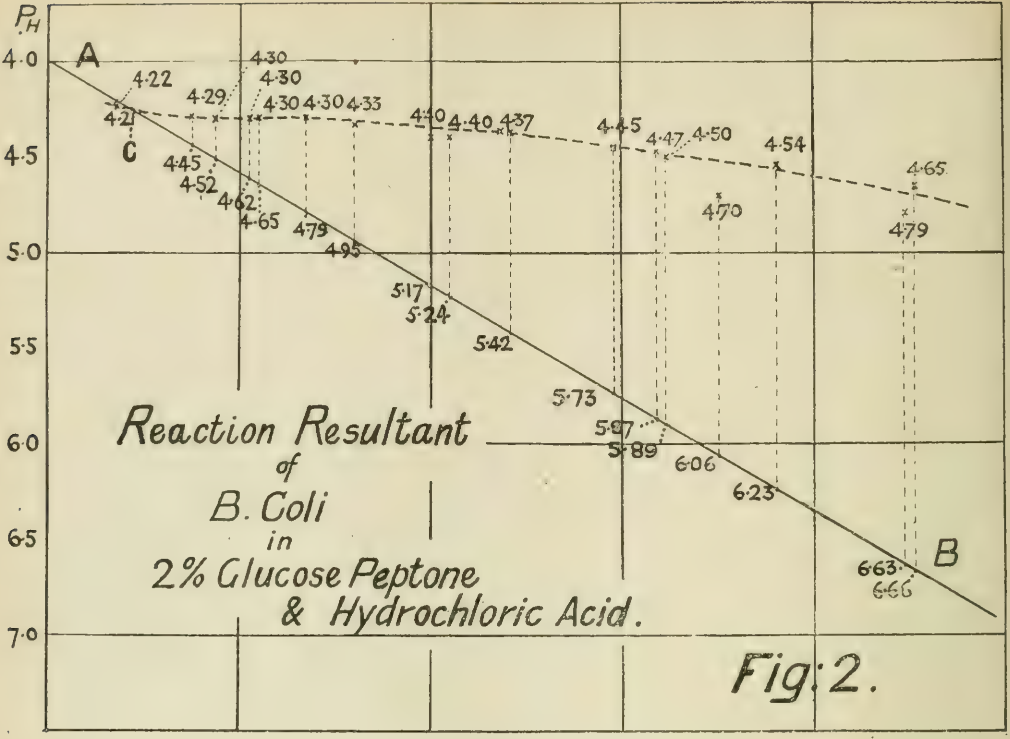


Fig: 2.

Cf. Table I.

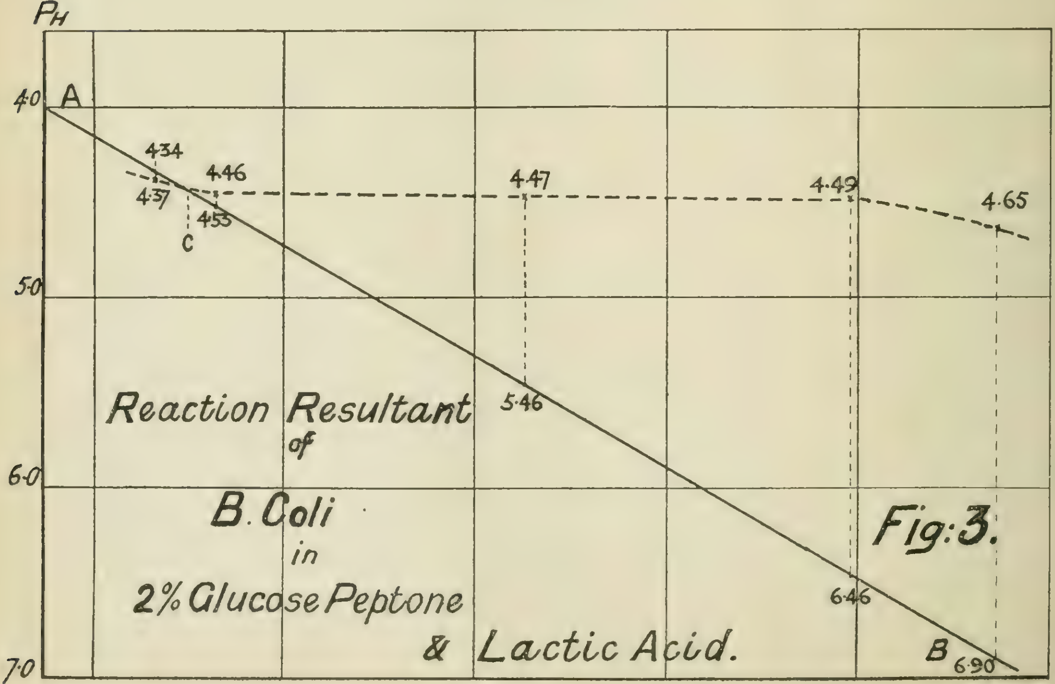
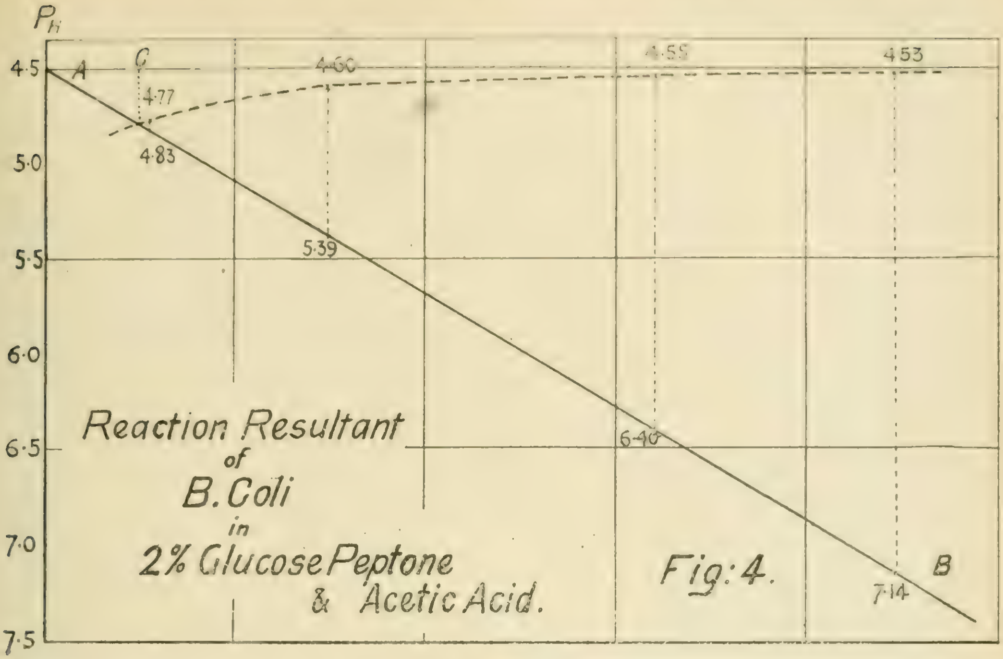
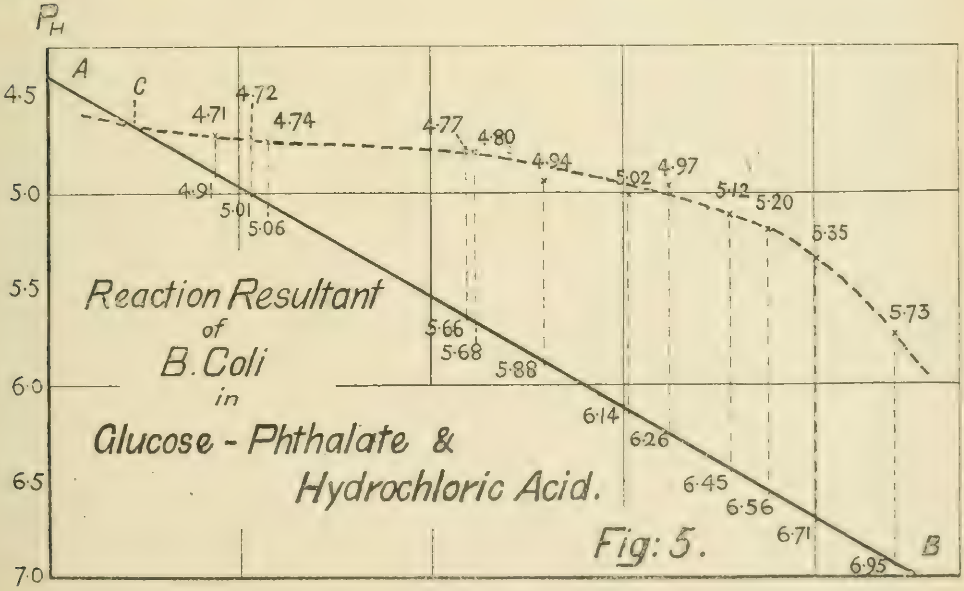


Fig: 3.

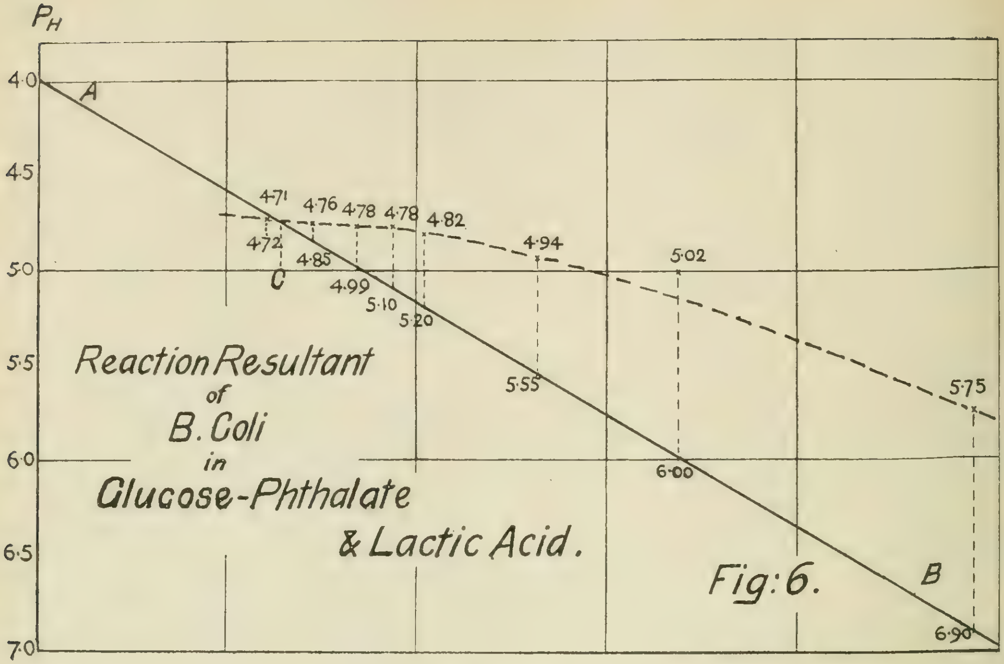
Cf. Table II.



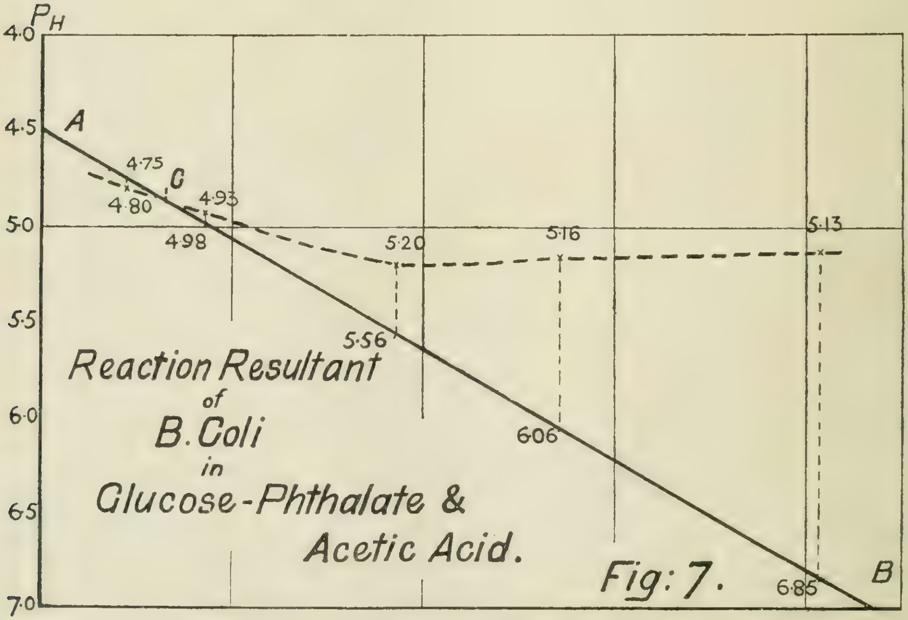
Cf. Table III.



Cf. Table IV.



Cf. Table V.



Cf Table VI.

which no acid has been added are considered, it is evident that any given strain of *B. coli* causes a higher final acid reaction in peptone than in phthalate of similar initial reaction: *e.g.* H_4 fermenting in peptone to which no acid has been added, causes a rise of P_H from 6·66 to 4·65, while it produces in phthalate of initial $P_H = 6·91$ a final reaction of $P_H = 5·73$.

All the available evidence therefore leads to the supposition that *B. coli* ferments more strongly in glucose peptone than in glucose phthalate, whether the media be devoid of acid, or whether equivalent amounts of the same acid have been added to each.

4. An examination of Tables I to VI and of the curves in Figs. 2 to 7 (which represent the reaction resultants in the different media, and in the presence of various acids), proves conclusively that the final acid reaction produced by the fermentation of *B. coli* is by no means a "physiological constant." It cannot be doubted that the final reaction reached in any given experiment is dependent upon the initial reaction of the medium in which the organism is allowed to ferment. In Figs. 2 to 7 the forms of the curves obtained are evidently dependent upon the nature of the acid added to the medium, and also to a less extent upon the nature of the medium itself. The exact value of the acid reaction at which fermentation is completely inhibited is more conveniently discussed in connection with the next question.

5. It is evident that for any given medium there is a certain definite acid reaction at which the growth of *B. coli* is completely inhibited. It is possible to define this "critical point" within very narrow limits. In the figures of the reaction curves this point is represented by C, the point at which the curve representing the final reactions obtained in the medium cuts the axis along which the initial concentrations are plotted. The critical value may also be approximately taken in any given case as the mean of the values obtained for the final acid reactions and the lowest acid reaction of the medium which completely inhibits fermentation. These values are shown in the following table:

TABLE VIII.

Medium	Acid	Most acid reaction permitting growth	Least acid reaction inhibiting growth	Mean critical point (Calc.)	Critical point (Curve)
Glucose peptone	Hydrochloric	4·30	4·25	4·27	4·25
	Lactic	4·52	4·42	4·47	4·43
	Acetic	4·77	4·60	4·68	4·78
Glucose phthalate	Hydrochloric	4·71	4·38	4·55	4·67
	Lactic	4·76	4·71	4·73	4·74
	Acetic	4·88	4·78	4·83	4·85

It will be seen that columns 5 and 6 of the above Table define, within very narrow limits, the critical point for *B. coli* fermenting in the given media and acids.

Although all strains of a particular variety, and both the "human" and "bovine" varieties of *B. coli* appear to react similarly in the presence of a given acid and medium it is evident that, quite apart from the influence upon the acid reaction exerted by the particular medium, there is a small but definite result due to the specificity of action of the added acid. The titration curves of the different acids in any given medium reveal, as has already been noted, but little specificity of action; the final reactions obtained appear to be a measure of the true acidity produced by the acid. When the reaction resultants are examined a much greater specific action of the acid is visible.

The results obtained in the foregoing experiments suggest that in any given medium "a strong" or highly dissociated acid (*e.g.* hydrochloric or lactic) permits a higher final acid reaction before complete inhibition of fermentation takes place. When however the mass of acid introduced is considered, it is found that the strong acids are more strongly inhibitory than is the "weaker" or less dissociated acetic acid, *e.g.* in glucose peptone the addition of an amount of hydrochloric acid sufficient to produce an acidity equivalent to 0·0180 N completely inhibits growth, the final acid reaction reached being $P_H = 4{\cdot}30$ while the acid constitutes 0·0657 per cent. of the mass of the solution. With lactic acid growth ceases at $P_H = 4{\cdot}47$ when the acidity is 0·0180 N and the acid constitutes 0·1637 per cent. of the mass of the solution. With acetic acid although the final acid reaction at which fermentation is inhibited is only $P_H = 4{\cdot}77$, the acid added to the medium is equivalent to an acidity of 0·0240 N and is 0·1440 per cent. of the mass of the solution. It is obvious that the mixture of medium and hydrochloric acid contains the least, that of medium and acetic acid the greatest, and that of medium and lactic acid an intermediate mass of undissociated acid. Pending an examination of the results obtained with other acids and media it would be premature to make a definite statement, but the results already obtained certainly suggest, that, when equivalent quantities are considered, highly dissociated acids are more effective in inhibiting the growth of *B. coli* than are those which are less dissociated. Further the final reaction reached by the culture depends rather upon the true acidity produced than upon the mass per cent. of acid present in the medium.

Examination of the forms of the curves expressing the reaction resultants of the various mixtures of media and acids reveals further signs of specificity.

While for hydrochloric and lactic acids the final reactions of the media become more acid, as the initial acid reaction of the medium increases, with acetic acid the reverse is obtained. For the former acids, it is evident that the amount of acid produced in the mixture is not rapidly diminishing in proportion to the rise of initial acid reaction, while with acetic acid a rise of initial acidity is accompanied by a marked diminution of acid formation.

It must be concluded that no attempts at diagnosis of the varieties of *B. coli* by means of their final reactions in a given medium can safely be made, since all strains and both varieties of the organism appear to react in the same manner when exposed to similar conditions. Also the final reaction attained is dependent upon the medium used, and the nature and amount of acid added to it.

In nearly every experiment it was found that at or near the expiration of a period of 168 hours from inoculation the P_H of the cultures showed a slight decrease of acidity. Possibly this is caused by the accumulation of metabolites produced during fermentation, and the phenomenon is graphically expressed in those figures in which the reaction resultant curve crosses, and passes to the left of, the initial reaction axis *AB*.

Table IX. *Glucose phthalate + hydrochloric acid, inoculated with* H_4 18 *hours old.*

				P_H						
Tube	*N*/5 acid per 10 cc. solution	Result	Time in hrs.	Initial	4	8	24	48	72	168
1	0	+		7·11	6·89	5·60	5·60	5·60	5·65	5·70
2	0·3	+		6·80	6·73	5·43	5·41	5·27	5·11	5·11
3	0·4	+		6·69	6·64	5·36	5·20	5·16	5·07	5·09
4	0·5	+		6·52	6·50	5·29	5·21	5·13	5·05	5·06
5	0·7	+		6·27	6·27	5·75	5·34	5·27	5·04	5·01
6	0·8	+		6·13	6·13	5·30	5·01	5·13	4·97	4·97
7	0·9	+		5·95	5·95	5·04	5·03	5·01	4·94	4·97
8	1·0	+		5·77	5·77	5·38	4·94	5·04	4·89	4·94
9	1·5	+		5·01	5·01	5·01	4·90	5·85	4·75	4·75
10	1·8	+		4·96	4·96	4·90	4·82	4·68	4·68	4·68
11	2·4	–		4·41	4·41	4·41	4·41	4·41	4·42	4·42

6. A number of "retardation" experiments were performed, and it was conclusively proved that in addition to the total inhibition of growth produced by the addition of an acid to a medium, smaller quantities of acid caused a well-marked retardation of the fermentation of *B. coli*. For any given acid and medium an increase in the amount of acid added resulted in a longer period of retardation before the solution reached its final acid

reaction. With either glucose peptone, or glucose phthalate, to which no acid had been added, signs of fermentation were always visible within four hours of inoculation, but when acid insufficient to produce total inhibition of growth was added the period of retardation was sometimes prolonged to nearly 48 hours. Table IX shows the results obtained in a typical "retardation" experiment.

SUMMARY.

1. All strains of *B. coli*, whether of human or of bovine origin behave similarly when exposed to similar conditions.

2. The degree of acidity of the final reaction produced by a culture of *B. coli* cannot be used for diagnostic purposes. This value is not a "physiological constant," but depends upon:

(*a*) The initial H-ion concentration of the medium in which fermentation occurs.

(*b*) The composition of the medium—especially the degree to which it is "buffered."

(*c*) The nature of the acid used to produce the initial reaction of the medium.

3. When the amount of acid added to a medium is insufficient completely to inhibit the fermentation of *B. coli* therein, a definite latency of growth results. For any given medium, a greater initial addition of acid causes a longer latent period.

4. Each acid appears to have a specific effect in inhibiting the growth of *B. coli* in a given medium, highly dissociated acids being more strongly inhibitory than those undergoing a less degree of dissociation.

5. For a mixture of any given medium and acid there appears to be a definite "critical point," beyond which the slightest rise in the degree of acidity results in a complete inhibition of the growth of *B. coli*. The critical points approximately determined as a result of the foregoing experiments are:

2 per cent. glucose peptone + hydrochloric acid $P_H = 4{\cdot}27$
,, ,, ,, lactic ,, $P_H = 4{\cdot}47$
,, ,, ,, acetic ,, $P = 4{\cdot}68$

Glucose + phthalate + hydrochloric acid $P_H = 4{\cdot}55$
,, ,, lactic ,, $P_H = 4{\cdot}73$
,, ,, acetic ,, $P_H = 4{\cdot}83$

The specificity of action of the acids in these mixtures is also illustrated by the form of the reaction-resultant curve obtained in any given case.

In conclusion I wish gratefully to acknowledge my indebtedness to Captain G. G. L. Wolf, R.A.M.C., at whose suggestion this research was undertaken, for much valuable advice and guidance during the prosecution of the work.

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XXXI. CARNOSINE, CONSTITUTION AND SYNTHESIS.

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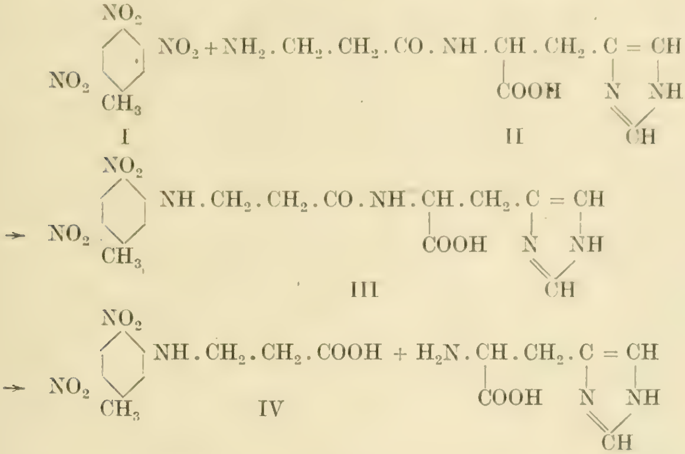
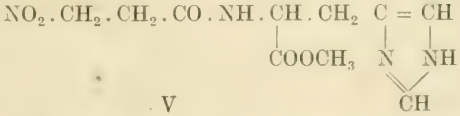
While engaged on work relating to the physiological behaviour of the trinitrotoluenes we observed that the β- and γ-isomerides condense with amino-acids on boiling in dilute alcoholic solution; the amino-acid becomes attached to the benzene ring by its amino-group, in replacement of a reactive nitro-group, which is eliminated. This reaction does not occur with imino-groups, whether present in a glyoxaline ring or in peptides, but does take place with the free amino-groups of peptides. The resulting compounds which are therefore *N*-dinitrotolylamino-acids resist boiling with concentrated hydrochloric acid and hence this reaction provides a suitable means of determining the constitution of peptides, for it is only necessary to combine the peptide with β- or γ-trinitrotoluene and hydrolyse the reaction product, when only those amino-acid radicles which originally had free amino-groups remain attached to the benzene ring, and these can therefore be identified. We applied this reaction to the dipeptide carnosine. Gulewitsch [1911] has shown that this base, one of the most important of those occurring in muscle extracts, is an anhydride of histidine and β-alanine, but whether β-alanylhistidine or histidyl-β-alanine, remained undecided.

This question had formerly occupied one of us in conjunction with Dr A. J. Ewins, and some experiments on the hydrolysis of deaminocarnosine, which the latter carried out in 1914, and has kindly allowed us to quote below, indicated that carnosine is β-alanylhistidine. It was, however, impossible to isolate the deaminocarnosine in a pure condition or to obtain more positive evidence of the deamination by isolating β-hydroxypropionic or acrylic acid after hydrolysis. The condensation product of γ-trinitrotoluene with carnosine on hydrolysis furnished dinitrotolyl-β-alanine in a pure condition, without any trace of the corresponding histidine compound, thus proving carnosine to be β-alanylhistidine and this result was communicated to the Biochemical Society at a meeting at the Lister Institute in December, 1917.

The reactions involved may be formulated as follows:


NO₂
NO₂ + NH₂ . CH₂ . CH₂ . CO . NH . CH . CH₂ . C = CH
NO₂
CH₃
COOH N NH
CH
I
II
NO₂
NH . CH₂ . CH₂ . CO . NH . CH . CH₂ . C = CH
→ NO₂
CH₃
COOH N NH
CH
III
NO₂
NH . CH₂ . CH₂ . COOH + H₂N . CH . CH₂ . C = CH
→ NO₂
CH₃
COOH N NH
CH
IV

γ-Trinitrotoluene is 2 : 4 : 5-trinitrotoluene, in which the 5-nitro-group is labile, as shown by the production of 2 : 4-dinitrotoluene by the action of ammonia, diazotising and boiling with alcohol. Its condensation product (III) with carnosine (II) is a carboxylic acid and (owing to the presence of the glyoxaline ring) also a base; it is therefore insoluble in ether. It is hydrolysed by boiling acids to IV, a carboxylic acid without basic properties, and soluble in ether, which was found to be identical with the substance obtained by condensing β-alanine with γ-trinitrotoluene. This result encouraged us to attempt the synthesis of carnosine. The action of ammonia on β-chloropropionylhistidine (prepared like the α-bromo-isocaproyl derivative of Fischer and Cone [1908]) did not yield the desired result. We therefore condensed histidine methyl ester with β-nitropropionyl chloride, and reduced the resulting product (V). After hydrolysis of the



V

ester grouping this yielded carnosine, which we eventually isolated with considerable loss as the copper salt and identified. Our single experiment on a small scale yielded only 19 mg. of the copper salt, and being mainly occupied with questions of a more practical kind, we did not repeat the experiment on a larger scale. Subsequently our attention was called to a paper by Baumann and Ingvaldsen [1918] who had also determined the constitution of carnosine and obtained the synthetic base in much larger quantity and in the free state. As our methods both of establishing the constitution, and of synthesis, differ from those given by Baumann and Ingvaldsen, we consider it advisable to publish our rather incomplete experiments.

A. CONSTITUTION.

Experiments by Dr A. J. Ewins (1914). 0·1 g. of carnosine was kept for two days at room temperature with one molecular proportion of silver nitrite and of hydrochloric acid. After filtration from silver chloride, a small amount of silver was removed from the filtrate by hydrogen sulphide. The solution was then concentrated to a small bulk and alcohol was added, when a gum separated. This was dissolved in concentrated hydrochloric acid and boiled for 6 hours, then evaporated to dryness. After redissolving in a little water and filtering from an amorphous impurity, the filtrate on standing deposited large colourless crystals of histidine dihydrochloride, M.P. 260°. The salt gave a picrate, M.P. 90–93°. In another experiment with 1 g. of carnosine and $1\frac{1}{2}$ molecular proportions of silver nitrite and hydrochloric acid numerous attempts were made to crystallise the deaminocarnosine, without success; nor did it yield a crystalline phosphotungstate. The yield of histidine dihydrochloride was from the main bulk 0·55 g. or 55 per cent. of the theory.

Action of γ-trinitrotoluene on carnosine. 0·5 g. of γ-trinitrotoluene and 1·0 g. of carnosine (two mols.) were dissolved in slightly diluted alcohol and boiled under a reflux condenser for one hour. As in all similar cases the liquid soon became deep yellow in colour. After evaporation on the water-bath, absolute alcohol was added and the evaporation was repeated to remove the small amount of water present. A bright yellow amorphous solid separated, very little soluble in absolute alcohol, even on boiling, but readily soluble in water. When damp with alcohol it was very deliquescent, but no longer so after washing with dry ether. This substance may be regarded as the carnosine salt of the condensation product of carnosine with γ-trinitrotoluene (III). It was dissolved in water and acidified with acetic acid when the condensation product itself was thrown down as a bright yellow precipitate, resembling the corresponding product from histidine and γ-trinitrotoluene. It was quite insoluble in ether, very little soluble in absolute alcohol and boiling water, much more readily in boiling 50 per cent. alcohol. On concentrating its solution in the latter solvent it separated in yellow granules but could not be obtained distinctly crystalline.

Hydrolysis of the condensation product; 0·3 g. was boiled for 5 hours with 30 per cent. aqueous sulphuric acid under a reflux condenser, when all but a trace of dark coloured resinous material had dissolved. The liquid was extracted with ether which removed a yellow substance. The ethereal solution was extracted with a small quantity of dilute ammonium carbonate and the resulting deep yellow alkaline extract acidified with sulphuric acid when a yellow solid separated. The latter crystallised from hot water or dilute alcohol in tufts of small, bright yellow needles, melting at 166°. The substance was evidently not the condensation product of γ-trinitrotoluene with histidine, which is insoluble in ether and decomposes violently at 265°, but rather the condensation product of γ-trinitrotoluene with β-alanine. This was synthesised and isolated in a similar manner, and found to be identical with the substance from carnosine. A mixture of the two melted at 166°. The synthetic compound was analysed.

0·1291 g.; 0·2102 CO_2; 0·0500 H_2O. C = 44·4, H = 4·3.

Calc. for $C_{10}H_{11}O_6N_3$. C = 44·6, H = 4·1.

B. SYNTHESIS.

We first attempted the synthesis in the way used by Fischer and Cone [1908] for the synthesis of leucylhistidine, and accordingly acted on histidine methyl ether with β-chloropropionylchloride. The crude condensation product, which could not be crystallised, was treated with ammonia at various temperatures: this did not result in the formation of any appreciable quantity of carnosine, but apparently of an unsaturated compound. With copper carbonate we obtained indications of carnosine copper salt, but the use of β-chloropropionyl chloride had to be abandoned. Baumann and Ingvaldsen found the same difficulty but were successful when they used the chloride of β-iodopropionic acid. We met this difficulty by using the chloride of β-nitropropionic acid, and reducing the compound formed. From glycerol we prepared successively glyceric acid, β-iodopropionic acid and β-nitropropionic acid, the last named according to Lewkowitsch [1879]. β-Nitropropionyl chloride had not been prepared hitherto: we heated the acid with excess of thionyl chloride on the water-bath for two hours, removed the excess of thionyl chloride by gently warming in a vacuum, and then distilled the residue in small quantities at a time by plunging the flask evacuated to

10 mm. into a metal bath previously heated to 160°. The object of this precaution was to limit the duration of heating, as otherwise the chloride is apt to decompose. We thus obtained an almost colourless liquid, B.P. 123°/10 mm.

0·1425 g.; 0·1511 AgCl. Cl = 26·2
Calc. for $C_3H_4O_3NCl$. Cl = 25·8.

The β-nitropropionyl chloride was next condensed with histidine methyl ester. 2 g. of the acid chloride in 20 cc. of dry chloroform was added gradually, with vigorous shaking, to a chloroform solution of rather less than two equivalents of the ester, cooled in a freezing mixture. As soon as the mixture ceased to give an alkaline reaction, the addition was discontinued and the chloroform solution of the condensation product was decanted from the histidine methyl ester monohydrochloride, which had separated. It was found impossible to isolate the condensation product itself since it decomposed on gently warming the solution, and quite appreciably during a few hours at ordinary temperatures. It was therefore at once reduced, by adding excess of stannous chloride in dilute hydrochloric acid to the above mentioned chloroform solution; the chloroform was removed by very gentle warming under reduced pressure. The temperature was then gradually raised and finally maintained at 100° for one hour. After cooling, the tin was removed as sulphide, and the filtrate was made slightly alkaline and kept over night to hydrolyse the ester. On evaporation to dryness the residue, consisting largely of sodium chloride, was extracted with methyl alcohol, which was distilled off, leaving a viscid residue of crude carnosine. As it could not be crystallised, we precipitated with phosphotungstic acid and obtained on regeneration 0·5 g. of a base, which on digestion with copper carbonate gave a deep blue solution. The latter was concentrated and deposited first an amorphous copper salt which was removed from time to time. Finally, on keeping the concentrated mother liquor for some days, ill-formed six-sided crystals separated, which were recrystallised and then yielded deep blue hexagonal plates, characteristic of the copper salt of carnosine. We finally only obtained 19 mg. of the pure salt. The recrystallisation is very troublesome and attended with great loss, as was pointed out by Gulewitsch and Amiradzibi [1900]. Perhaps we would have done better to attempt the crystallisation of the nitrate of the base regenerated from the phosphotungstate. The copper salt was dried *in vacuo* over sulphuric acid for some months, and was then analysed by Pregl's micro-Kjeldahl method [see Abderhalden, 1912].

I. 2·85 mg.; 2·65 cc. $N/70$ HCl. N = 18·6,

II. 5·7 mg.; 5·0 cc. $N/70$ HCl. N = 17·5.

For the sake of comparison natural carnosine copper salt was also analysed.

3·25 mg.; 3·0 cc. $N/70$ HCl. N = 18·5.

Calc. for $C_9H_{14}O_3N_4 . CuO$. N = 18·3.

We also determined the rotation. It was considered inadvisable to attempt the removal of the copper from so small a quantity. Hence we merely dissolved the salt in excess of dilute acid.

9 mg. Cu salt in 200 mg. 13 per cent. sulphuric acid gave a pale blue solution containing 6·6 mg. carnosine.

$$c = 3{\cdot}3 \text{ per cent.};\ l = 0{\cdot}5 \text{ d.m.};\ \alpha_D = +\,0{\cdot}15^\circ;\ [\alpha]_D = +\,9^\circ.$$

Gulewitsch [1913] gives for carnosine nitrate dissolved in excess of nitric acid, $[\alpha]_D = +\,12^\circ$ to 13°.

We desire to express our thanks to Dr J. C. Drummond, of the Cancer Hospital, for the use of a micro-polarisation apparatus and kind help in the above determination.

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XXXII. ACCESSORY FACTORS IN THE NUTRITION OF THE RAT.

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The researches of McCollum and Davis have revealed the fact that rats cannot grow or exist on an artificial diet unless two principles as yet chemically unknown are present. These were designated by McCollum and Kennedy [1916] as fat-soluble A and water-soluble B. The former is associated in nature mostly with fats of animal origin; the latter is soluble in water. McCollum and Kennedy have further found that the water-soluble B if not identical with the antineuritic factor is at least closely associated with it, and this has been confirmed by Drummond [1917]. More recently McCollum and Pitz [1917] and Pitz [1918] have expressed the view, based on observations made on guinea-pigs and rats, that scurvy is not a disease produced by the deficiency of an accessory factor and that the scorbutic condition produced in guinea-pigs by dietetic means is due to other unfavourable points in the diet. In a previous communication [1918, 2] we have already discussed their experiments on the guinea-pigs and have shown that their conclusions were not warranted by the experimental evidence brought forward in their papers. We hold the view that scurvy is a disease brought about by the dietetic deficiency of a principle distinctly different in its physical and other properties from the antineuritic and the fat-soluble A factors. It is evident from the extensive researches of Osborne and Mendel, and McCollum and his collaborators that by omitting the addition of antiscorbutics to their purified diet, no scorbutic lesions can be produced in rats, such as are found in human subjects, monkeys and guinea-pigs, when they subsist on diets deficient in antiscorbutics. Guinea-pigs when put on oats, bran and autoclaved milk show scorbutic symptoms 14–21 days after the commencement of the experiment. Our own observations on monkeys [1919] show that these animals manifest scorbutic lesions after 2½–3 months' subsistence on a diet free from antiscorbutics. As regards human subjects there appear to be a few apparently reliable clinical records of cases in which, owing to adverse circumstances, the subjects have been entirely deprived of antiscorbutics for a considerable time. Judging from these, 6–8 months seems to be the time necessary for the subject to exist on a deficient diet before the disease is well declared. In perusing the literature one finds, however, that rats have been kept by various experimenters for a considerable length of time on scorbutic diets without showing any signs of scurvy. The experiments we are about to describe show nevertheless that rats existing on a scorbutic diet, although capable of gaining in weight and reproducing themselves, without any apparent manifestation of pathological symptoms for months, do not thrive so well as animals which have their diets supplemented with an antiscorbutic. This suggests that although rats are not very susceptible to scurvy they cannot absolutely dispense with antiscorbutics without restriction of their normal development.

EXPERIMENTAL.

Our investigations were carried out on several groups of rats receiving a purified basal diet of starch, caseinogen and inorganic constituents and the various accessory factors. The basal diet was made up as follows:

Starch	75	per cent.
Caseinogen	20	,,
Salts	5	,,

The salt mixture was the same as that employed by McCollum and his collaborators in their researches and had the following composition:

NaCl	1·73 g.	$CaH_4(PO_4)_2 2H_2O$	5·40
$MgSO_4$ (anhydr.) ...	2·66	Calcium lactate ...	13·0
$NaH_2(PO_4)H_2O$ ...	3·47	Ferric citrate ...	1·18
K_2HPO_4	9·54		

The fat-soluble A factor was supplied in the form of butter-fat purified by centrifuging the melted fat and decanting the supernatant layer. The antineuritic factor was introduced by adding autolysed yeast. The antiscorbutic was added in the form of lemon juice from which the citric and other organic acids had been removed by the method we have already described in a previous communication [1918, 1].

For the sake of convenience we shall designate in this paper the diets of the various groups by the accessory factors they contain, using the letters

A, B, and C for the fat-soluble A, antineuritic, and antiscorbutic factors respectively. The rats were kept separately in cages; couples belonging to the same group were brought together only occasionally for mating purposes. The food was supplied to them fresh in balls every day and the remains of the previous day were weighed daily. The drinking water was changed twice daily.

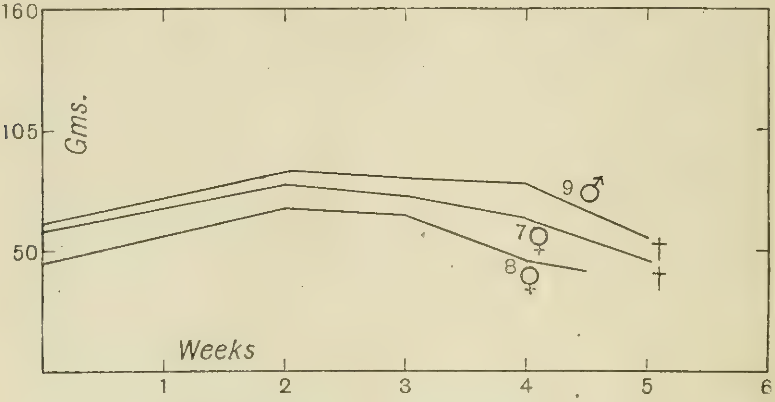

Fig. 1. Weight curves of 3 rats on an AC diet.

Fig. 1 represents the weight curve of 3 rats kept on an AC diet. The daily ration of each of these rats was made up as follows:

Basal mixture	10 g.
Antiscorbutic equiv. to lemon juice ...	5 cc.
Centrifuged butter fat	1·8 g.
Olive oil	1·6 g.

The above ingredients were incorporated in a single ball, the olive oil being added in order to obtain a convenient consistency in the paste. From $\frac{1}{2}$–$\frac{3}{4}$ of the daily ration was consumed by the animals.

It is seen from the weight curves that none of the animals has gained appreciably in weight during any period of the experiment. This becomes more marked as the rats were at an age when development under normal conditions progresses rapidly. After about three weeks the animals commenced losing in weight and died five weeks after the commencement of the experiment. Rat No. 8 was killed by the buck after $4\frac{1}{2}$ weeks when left with it in a cage for mating purposes. Rats Nos. 7 and 9 showed decided neuritic lesions before death. Rat No. 7 first showed marked paralysis in the hind limbs four days before death. This condition became gradually worse. Rat No. 9 showed even more striking symptoms. The following was its condition three days before death[1]. Rotated as ingoing corkscrew. Position of left ear approximated left shoulder. Left eye to the ground. Tone increased on left side. Body was bent anticlockwise, *i.e.* concavity to the left. No circus movements observed. Animal was able to use fore and hind legs for grasping objects apparently without incoordination. Could not walk in a normal manner. It stood on a wide base. Difficulty appeared to be due to the increase in tone of muscles of limb. A day later the animal showed circus movements and died on the subsequent day. Portions of the sciatic nerves of rats No. 7 and No. 9, teased and treated with osmic acid, were examined histologically by Miss F. M. Tozer. In Miss Tozer's opinion the greater part of the nerve of rat No. 9 was sound. There were however a fair number of curiously eroded abnormal looking fibres concerning which in the absence of further evidence it is difficult to give a definite opinion. Their appearance is possibly suggestive of a very early stage of degeneration. The teased preparation of the nerve of rat No. 7 showed no degenerating fibres.

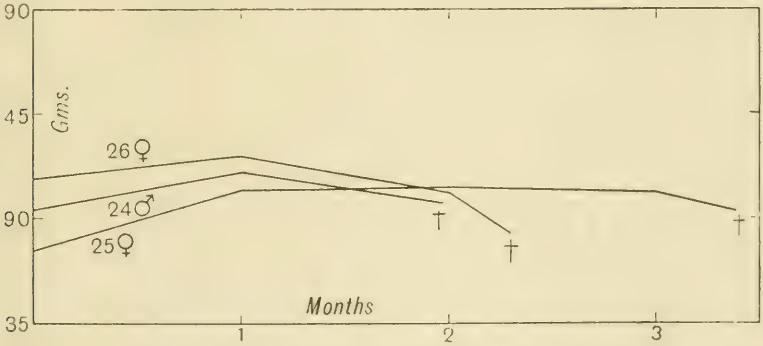

Fig. 2. Weight curves of 3 rats on a purified B diet.

Fig. 2 represents the weight curves of three rats fed on a B diet. The daily ration of these animals was as follows:

Basal mixture ...	...	12 g.	Water ...	...	2½ cc.
Autolysed yeast	...	5 cc.	Olive oil ...	...	2½ cc.

From ½–¾ of this ration was consumed by the animals daily. All these animals succumbed to the fat-soluble A factor deficiency within a period varying from 2 to 3½ months. During the first month they all gained in weight. Their weight then became more or less stationary, but the animals declined a little before death. Two of these rats, namely 24 and 26, showed xerophthalmia in acute form.

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[1] We are indebted to the late Dr F. E. Batten for this description of the condition of the animals.

That the antiscorbutic factor does not fulfil the same physiological function as the fat-soluble A factor is seen from Fig. 3 which represents the weight curves of three rats fed on a BC diet. The daily ration of this group was made up as follows:

Basal mixture	12 g.
Antiscorbutic equiv. to lemon juice	5 cc.
Autolysed yeast juice	5 cc.
Olive oil	0·75 cc.

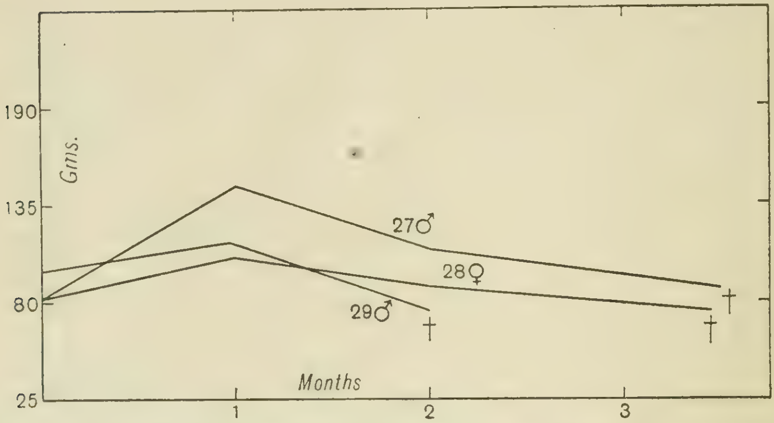

Fig. 3. Weight curves of 3 rats on a pure BC diet.

About three-quarters of the daily ration was consumed by the rats. The weight curves of this set do not differ much from those of the previous group. Rat 29 died two months and rats 27 and 28, 3½ months after the commencement of the experiment. They all developed xerophthalmia. It is evident from this experiment that the addition of an antiscorbutic to a diet lacking the fat-soluble A does not rectify the deficiency.

In the last two experiments the caseinogen employed was previously extracted with ether and alcohol. Experiments of the same character as these two were made in which the caseinogen had not been previously purified. The results obtained are of sufficient interest to be recorded. Fig. 4 shows the weight curves of a group of rats fed on B diet with unpurified caseinogen and Fig. 5 represents the same of a group of rats fed on a BC diet with unpurified caseinogen.

The general characters of these curves are the same as those of the animals fed on similar diets with purified caseinogen. The main difference lies in the prolonged period of time during which the animals lived on the experimental diet. After about two months, growth was entirely suspended and a decline in weight was observed towards the end of the experiments. Many of these animals displayed xerophthalmia. During the progress of the experiment the buck of each set was on several occasions mated with the does, but no litters were produced. This prolonged sustenance on a diet greatly deficient in the fat-soluble A factor afforded us the opportunity of observing certain marked histological changes in the *post-mortem* tissues of these animals which are of decided interest. We shall describe and discuss these results in another communication.

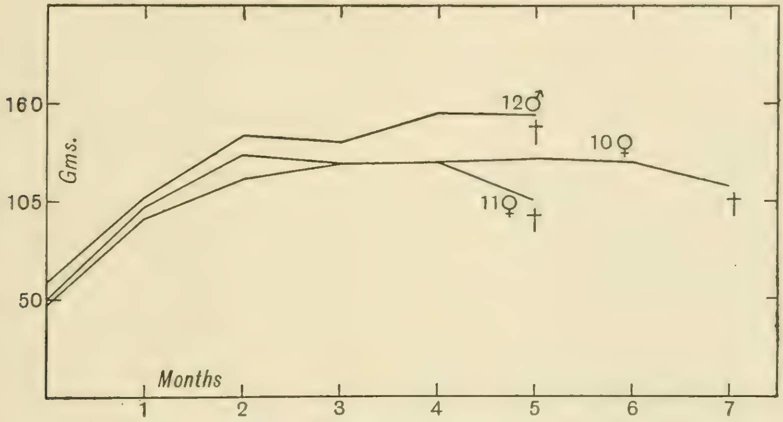

Fig. 4. Weight curves of 3 rats on a crude B diet.

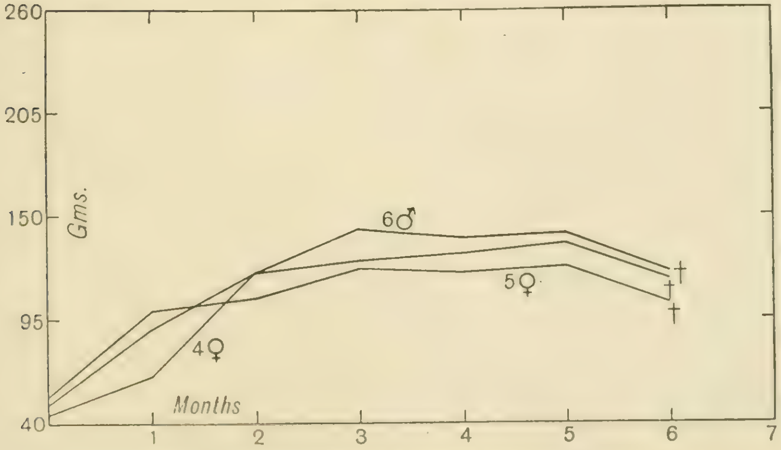

Fig. 5. Weight curves of 3 rats on a crude BC diet.

Two groups of rats were also fed on AB and ABC diets. These were made up as follows:

	AB	ABC
Basal mixture	10 g.	10·6 g.
Purified butter	1·85 g.	1·7 g.
Autolysed yeast	5 cc.	5 cc.
Olive oil	2·5 cc.	0·25 cc.
Antiscorbutic equiv. to lemon juice	—	5 cc.

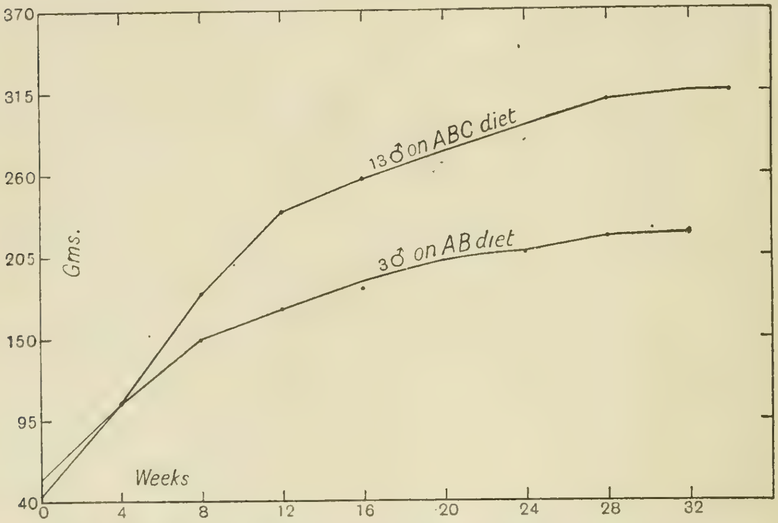

Fig. 6. Weight curves of 2 ♂ rats on ABC and AB diets respectively.

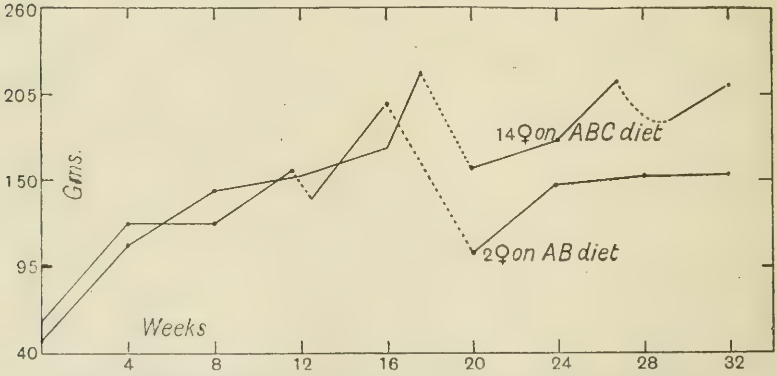

Fig. 7. Weight curves of 2 ♀ rats on ABC and AB diets respectively.
- - - - indicates that no weighings were made owing to the birth of a litter.

These two groups grew satisfactorily and reproduced themselves. The ABC group however flourished decidedly better than the AB group. This can be seen from Figs. 6 and 7 which represent the weight curves of animals belonging to those two groups. Fig. 6 represents the weights of the two respective bucks and Fig. 7 of the two does on the two diets. In both cases the difference in growth is decidedly marked. This observation does not appear to be accidental as similar observations have been made in this laboratory on other animals kept on similar diets and so far no deviation from the above behaviour has been recorded.

If animals kept on the AB diet do not develop to their fullest capacity without having the antiscorbutic added in their diet, it would appear that rats are susceptible to an antiscorbutic deficiency although they do not develop definite lesions. Further investigations will definitely decide this point.

SUMMARY.

1. An antiscorbutic does not fulfil the physiological function of the fat-soluble A when it replaces it in the diet of the rat.

2. The authors confirm the observation of McCollum and his colleagues and of Drummond that by depriving rats of the antineuritic factor a dietetic deficiency is brought about as a result of which neuritic lesions are produced in the animals and a fatal termination ensues if the diet is not rectified in time.

3. Rats subsisting on a diet containing the antiscorbutic as well as the water-soluble and fat-soluble A factors grow better than rats from the diet of which the antiscorbutic factor is absent.

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XXXIII. THE ANTISCORBUTIC VALUE OF CABBAGE. I. THE ANTISCORBUTIC AND GROWTH PROMOTING PROPERTIES OF RAW AND HEATED CABBAGE.

By ELLEN MARION DELF,

WITH AN APPENDIX

By FRANCES MARY TOZER.

ON THE HISTOLOGICAL DIAGNOSIS OF EXPERIMENTAL SCURVY.

From the Lister Institute of Preventive Medicine.

(*Received November 12th, 1918*)

The antiscorbutic properties of fresh vegetables are well known and cabbages of all kinds are especially valuable in this respect. At the present time, owing to the scarcity of fresh fruit, it is important that the best use should be made of any available green vegetables. Most vegetables require cooking in order to make them digestible and palatable enough for human consumption. It is known, however, that the antiscorbutic value of both vegetables and fruit is diminished by cooking, and the present investigation is an attempt to estimate the loss in the antiscorbutic properties of fresh cabbage after exposure to heat at various temperatures and for different intervals of time.

In order to have some basis of comparison it has been necessary to make preliminary experiments using varying amounts of fresh raw cabbage as the sole antiscorbutic in an otherwise adequate diet. Guinea-pigs were chosen as experimental animals and the standard diet selected was a mixture of rolled oats and bran *ad libitum*, and water. With small rations of cabbage (5 g. daily and under) 60 cc. of milk previously heated in an autoclave for one hour at 120° C. was also given daily. This was found to improve the nutritive value of the diet and encourage growth without influencing the onset of scurvy.

The Antiscorbutic Value of Raw Fresh Cabbage.

Experiments of Holst and Frölich [1912] indicate that normal growth of young guinea-pigs can be secured on a diet of fresh cabbage leaves and some kind of cereal food (oats, rice, rye bread or white bread). The change in body-weight relative to the initial weight varied considerably with the amount of cabbage given (see Table I). The only ration which gave both a fairly satisfactory growth and complete protection from scurvy was one of 30 g. daily, but probably younger animals would have made growth on a smaller ration. However, the animals were evidently not under very favourable circumstances, for out of 56 cases cited [Holst and Frölich, 1912, Table IV A], 27 animals died from illness—sometimes of an unknown character, but often from pneumonia or enteritis. Nearly complete protection from scurvy was given by a daily ration of only 1 g., but the animals survived for only a short time with much loss of weight, and in the further work of these authors on dried and heated vegetables, 30 g. of fresh cabbage daily was chosen as the standard ration.

Table I. *Summary of Results of experiments of Holst and Frölich with fresh cabbage* (*calculated from their paper* [1912, Table IV A, p. 64, and pp. 106–112]).

Ration given	Mean initial weight of animals in g.	No. of animals	Time of experiment in days	Total gain or loss in weight %	Result
30 g. white cabbage and oats and bran *ad lib.*	328	5	100–200	+33	No scurvy
15 g. white cabbage and white bread	507	3	33–105	−25	do.
10 g. white cabbage and white bread	475	4	46–113	−22	Traces of scurvy in one case
10 g. white cabbage and rice (husked)	676	4	28–72	−41	do. do.
25 g. white cabbage and oats and bran *ad lib.*	342	2	15–28	−50	No symptoms in one case, loose teeth in one case
1 g. white cabbage and oats and bran *ad lib.*	307	3	47–56	−50	No symptoms in one case, loose teeth in one case

In our experiments, care has been taken to give the animals the best conditions possible. The laboratory in which they were kept was well ventilated and warmed during the winter (60°–65° F.). Most of the animals were bred on the premises and were well fed and housed from the first. Shortly before starting on an experiment, the animals were placed in separate cages on a litter of peat and were kept under observation for a few days. If the general condition and the weight curve were satisfactory, the experiment was started when a suitable weight had been attained (about 330–350 g.) but doubtful animals were rejected for the purposes of these experiments.

In the case of animals under experiment, all the rations were given by myself or by fully trained assistants and a daily record was kept of the amount actually consumed. It was generally possible to ensure that the whole of the cabbage ration was eaten, hand feeding being introduced where necessary. All the cabbage was given as green leaf, avoiding both the thickest part of the midrib, and the whitish inner leaves of the heart. In this respect the cabbage used differs from that given by Holst and Frölich who used "white" cabbage (*i.e.* the winter cabbages with large, compact, white hearts) and did not apparently select definitely either the inside or outer green leaves. In our experiments with small rations, especial care was exercised in selecting only the healthy green parts of the turgid leaf to ensure uniformity as far as possible.

Clean water or milk, as the case might be, was given twice daily, and almost every day the animals were allowed to run about on bench or table for exercise. We believe that this regular exercise, even when only for brief periods of time, has a very beneficial effect on their general health and well being. It also allows observation of any inactivity or lameness which is a frequent precursor of the onset of scurvy. For the most part the animals were remarkably healthy; out of the 27 animals on fresh cabbage quoted in Table II only one died from an unknown cause before the completion of the three months of the experiment. In spite of every precaution, however, and the daily disinfection of all the vessels used for feeding, some unexplained deaths occurred occasionally, especially on the smaller limiting ration of cooked cabbage. This may be caused by lowered resistance to chance infection resulting from an insufficient margin of antiscorbutic in the diet to ensure perfect robustness.

Animals of about 330 g. initial weight have been selected as far as possible and the usual duration of an experiment was 90 days. The cereal food given throughout was a mixture of rolled oats and bran which was readily and often greedily eaten. The animals were weighed three times weekly and carefully examined every 5 or 6 days, or oftener when the onset of scurvy was expected.

The symptoms to be found in scorbutic animals have been described in a communication by Chick, Hume and Skelton [1918], but may be recapitulated here. They are: (1) Soreness of joints. (2) Lameness, with or without general inactivity. (3) The adoption of various typical "scurvy positions" when in repose. In these positions, the limb affected is often held up and in the "face-ache" scurvy position the head is lowered and the neck stretched out (see Plate I, figs. 2 and 3). Post-mortem examination of animals which have frequently adopted the "face-ache" position shows that the teeth are loose and the jaws so fragile as to break with the slightest pressure. (4) Severe cases of long standing have swollen knees often fractured at the epiphysial junction of the tibia, the back limbs being then almost helpless. Photographs of some typical scurvy positions are shown in Plate I.

The post-mortem signs of scurvy are well known and comprise chiefly (1) haemorrhages in the subcutaneous tissue, muscles, periosteum or viscera, (2) fragility of bones, with fracture in severe cases, (3) ridges or swellings on the rib-junctions, (4) loosened teeth set in a fragile jaw and (5) wasted tissues. A systematic examination has been made by Miss F. M. Tozer of the minute structure of the rib-junctions of these and many other experimental animals. On the whole, her independent histological diagnosis falls into line with the symptoms observed during life and with the results of post-mortem examination. In general, it has been possible to distinguish histologically three principal stages in the course of the disease, which may be termed respectively incipient, definite and acute[1]. The incipient stage involves only slight histological abnormality (Plate II, fig. 2) corresponding to absence of definite symptoms in life and very slight evidence of scurvy or none at all at the post-mortem examination. Definite and acute scurvy implies marked disorganisation of the normal structure of the bone cartilage junction (in which growth appears to become arrested in chronic cases) and agrees usually with marked evidence of scurvy during life and at the post-mortem examination. Further details of these stages are given by Miss Tozer in Appendix I together with diagrammatic illustrations (Plate II, figs. 1–6).

Table II gives a summary of the results of the experiments with raw cabbage rations, fuller particulars being given in Table VII. With a 30 g. ration (Exp. 1) the amount of growth was much greater than that found by Holst and Frölich, although the average initial weights of the animals were very similar. The mean growth curve A (Fig. 1) obtained by averaging the body-weights from time to time of all the animals in Exp. 1 has been adopted as the standard of normal growth throughout this investigation. It is nearly identical with the mean curve of normal growth deduced by Chick, Hume and Skelton from the growth of twelve animals upon full

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[1] See note, p 446.

Table II. *Experiments with limiting rations of fresh cabbage* (see also Table VII).

Exp.	Ration of fresh cabbage g.	Number of animals	Mean initial weight g.	Total gain %	Time of exp. days	Symptoms in life	Post-mortem examination	Histology of rib-junctions	General conclusions
1	30	3	340	91	87–90	None	Not examined	Not examined	Complete protection
2	15	4	345	74	90	do.	No indications of scurvy	Normal	do. do.
3	5	3	323	26	90	Slight soreness	do. do.	Incipient scurvy	Protection
4	5	3	325	69	90	do.	do. do.	do.	do.
5	2·5	5	323	49	90	Soreness in 2 cases	Brittle leg bones in one case, brittle jaw in another	do.	do.
6	1·5	6	346	49	70–90	Soreness in 5 cases out of 6	Brittle bones in 2 cases	do.	do.
6 A	1·5 (citric acid)	4	330	70	90	Slight soreness after 49 days	2 normal, 2 slight symptoms	1 normal, 3 nearly normal	do.
7	0·5	4	331	18	67–90	Soreness, swollen joints	Three with fragile bones. All with rib-junctions ridged	1 normal 1 slight scurvy 1 chronic scurvy 1 severe scurvy	Scurvy in 3 out of 4 cases

In Expts. 1, 2, and 3 the rest of the ration consisted of water and rolled oats and bran *ad libitum*, the amount consumed daily being about 30 to 60 g. (see Table VII).

In Expts. 4–7 60 cc. milk autoclaved at 120° C. for 1 hour was substituted for the water.

normal diet. The average increase in body-weight is strikingly different in Expts. 1, 2, and 3, diminishing with the reduction of the cabbage ration, although the appetite was not impaired, and the grain consumed was not less (cp. Table VII and curves A, B and C, Fig. 1). In each case there was no symptom of scurvy during life, but the slight tenderness observed in the joints of animals fed on the 5 g. ration and the results of histological examination of their rib-junctions suggest a condition of incipient scurvy.

Since in these animals, growth was apparently limited by the extent of the cabbage ration apart from the incidence of scurvy, another series of animals were given a daily allowance of 60 cc. milk previously autoclaved for an hour at 120°, in order to improve the nutritive value of the diet. Previous experience of workers in this Institute has proved that the addition of this amount of autoclaved milk does not affect the onset of scurvy. Expts. 3 and 4 with a ration of 5 g. cabbage with and without milk, respectively, fully confirm this result. In both sets of animals a departure from the normal could be detected at histological examination of the rib-junctions, though in both sets the animals gave no marked signs of scurvy during life or at the post-mortem examination. The animals on the milk ration however grew much better and were heavier and in better condition throughout the experiment, which lasted three months.

No indications of scurvy were detected during life in most of the animals on 2·5 and 1·5 g. rations of cabbage to which autoclaved milk had been added (Expts. 5 and 6). The mean growth of these animals was less than that of those on the larger rations and the irregular growth on the smaller of these rations is probably significant (C and D, Fig. 2). On histological examination of the rib-junctions incipient scurvy was diagnosed in several cases. On the smallest ration of cabbage given, 0·5 g. (Exp. 7) no growth was made after the first 20 days (E, Fig. 2); three out of the four animals exhibited definite symptoms of scurvy and one died of acute scurvy after 67 days. There is little doubt that most young guinea-pigs would find this ration insufficient for maintenance of normal health. The limiting daily ration of fresh cabbage just protecting a young guinea-pig from definite symptoms of scurvy during life and maintaining health, upon the standard diet of oats and bran and autoclaved milk must therefore lie between 1·5 and 0·5 g. In the experiments of Holst and Frölich a ration of 1 g. fresh cabbage daily was found to give nearly complete protection from scurvy (Table I), and in the present communication this ration has been regarded as the probable minimum protective ration.

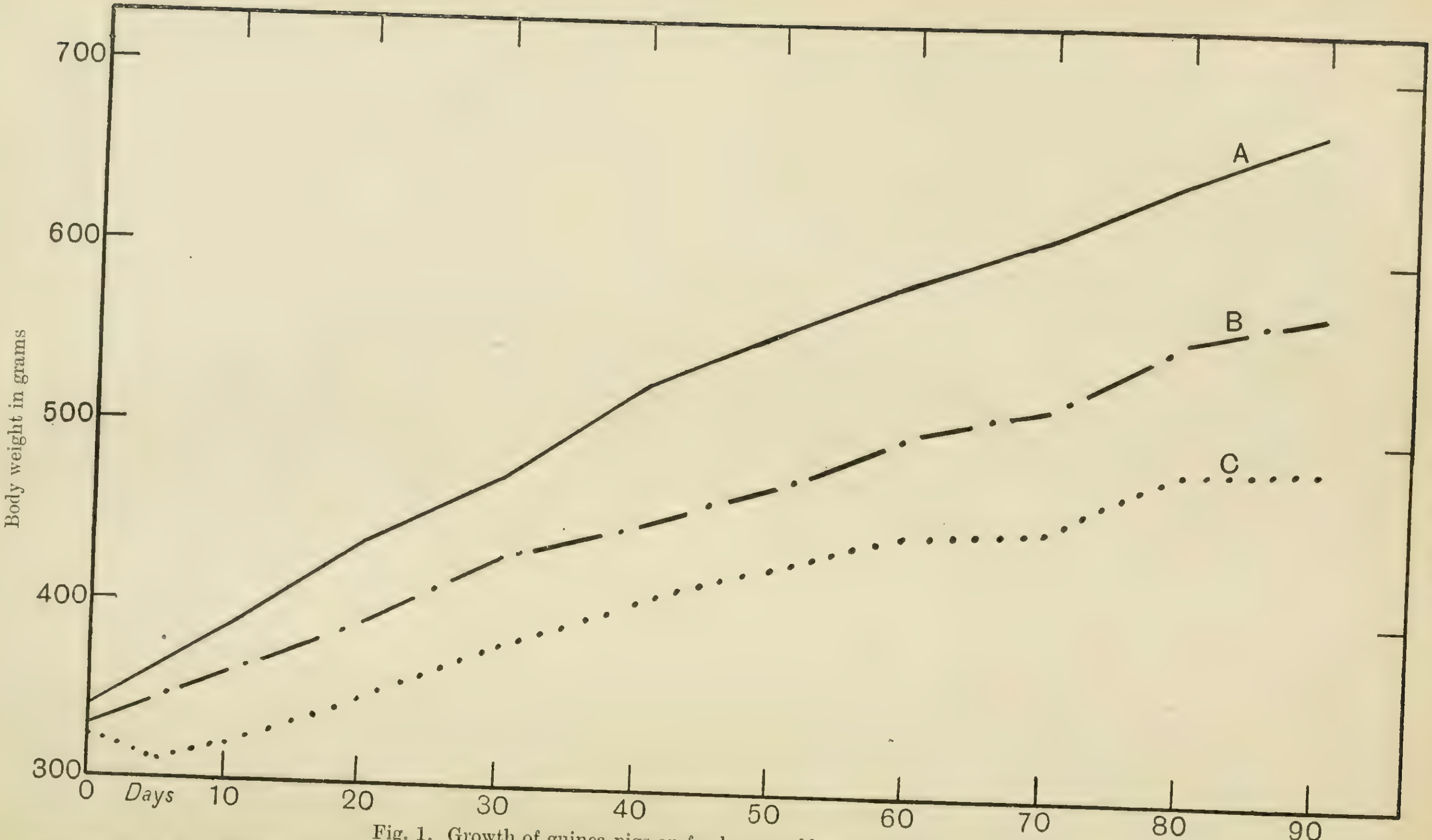

Fig. 1. Growth of guinea-pigs on fresh raw cabbage, oats, bran and water.

——— Curve A. Represents the standard growth curve of 3 animals upon a 30 g. fresh cabbage ration, oats, bran and water.

—·— ,, B. Represents the average growth of 4 animals upon 15 g. fresh cabbage ration, oats, bran and water (Expt. 1, Tables II and VII).

······ ,, C. Represents the average growth of 3 animals upon a 5 g. fresh cabbage ration, oats, bran and water (Expt. 2, Tables II and VII).

Represents the average growth of 3 animals upon a 5 g. fresh cabbage ration, oats, bran and water (Expt. 3, Tables II and VII).

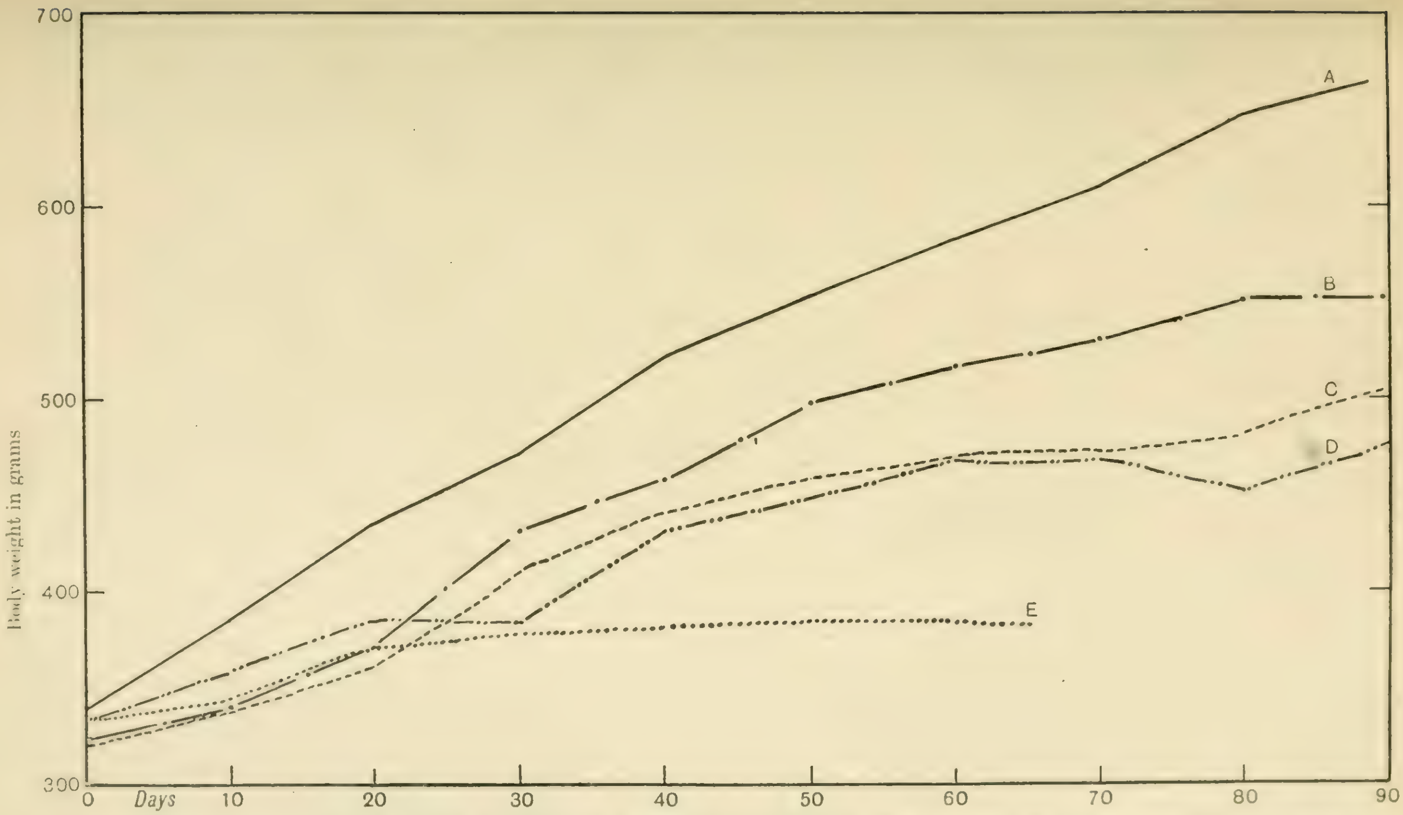

Fig. 2. Growth of guinea-pigs on fresh raw cabbage, oats, bran and autoclaved milk.

Mean weight curves of five groups of young guinea-pigs upon a diet of oats, bran, fresh cabbage and autoclaved milk daily. Each curve is obtained from the mean weights of all the animals in the experiment after definite time intervals (Tables II, VII, Expts. 1 and 4–7).

——— Curve A. Represents the standard growth curve of 3 animals upon a 30 g. cabbage ration (Expt. 1, Tables II and VII).
—•— ,, B. Represents the average growth curve of 4 animals upon a 5 g. cabbage ration with 60 cc. autoclaved milk.
- - - - - ,, C. ,, ,, ,, 4 ,, 2·5 g. ,, ,, ,,
—••—•• ,, D. ,, ,, ,, 4 ,, 1·5 g. ,, ,, ,,
•••••• ,, E. ,, ,, ,, 4 ,, 0·5 g. ,, ,, ,,

From this series of experiments we may therefore conclude:

(1) That normal growth on a ration of 30 g. fresh cabbage under the conditions of our experiments involves an increase of body-weight of about 100 per cent. in 90 days.

(2) That smaller rations involve a corresponding diminution in the growth rate without however causing any definite symptoms of scurvy to appear during life unless the ration is reduced to 0·5 g.

(3) That apparently satisfactory growth and health are obtained with rations from 1·5–5 g., if 60 cc. autoclaved milk daily is added to the diet, but suggestions of soreness during life, together with departure from the normal in the histology of the bone cartilage junctions point to a condition of incipient scurvy.

(4) That on a 0·5 g. ration well-marked symptoms of scurvy may be expected.

(5) That a 1 g. ration of fresh cabbage may be regarded as the minimum for protection from the symptoms usually diagnosed as scurvy.

The Antiscorbutic Value of Cooked Cabbage.

The only information available as to the effect of heat on the antiscorbutic properties of cabbage is contained in the paper of Holst and Frölich [1912] to which reference has already been made. Their general conclusion was that heat has a distinctly destructive effect and that this destruction is greater at 110° and 120° than at 100° for the same time of heating, viz. 30 minutes.

They used steam under pressure in an autoclave for heating the material at 110° and 120°, but at 100° the leaves were either boiled in slightly salted water or they were placed in an Erlenmeyer flask and heated for the required time, the leaves being slightly salted. The animals are said to have licked up the juice which exuded during heating, and also to have eaten their ration of cabbage: they appear to have refused to eat it when unsalted.

Table III gives a review of their results, the averages of the initial and final weights of the period of survival being calculated from their figures (H. and F., Table IV B, pp. 66–70). After being heated for ½–1 hr. at 100° a ration of 30 g. appears to have protected the animals almost completely from scurvy, but there was little or (in one set) no gain in weight as would have been expected on a corresponding ration of fresh cabbage. With 30 g. cabbage heated ½ hr. at 110° one animal out of three had clearly scorbutic symptoms (loose teeth, haemorrhages and disorganised rib-junctions) whilst all animals were similarly affected when the same ration was heated for 1 hr. at 120°.

TABLE III. *Summary of Experiments of Holst and Frölich with Cooked Cabbage.* [See H. and F., 1912, Table IV B, pp. 66–70.]

Diet	No. of animals	Mean time of life	Mean weight in g.		Result
			Initial	Final	
30 g. cabbage cooked 1 hr. in ½ % common salt at 100° C. and oats	5	87 days	351	352	Complete protection except in one case
30 g. cabbage steamed ½ hr. at 100° and white bread	3	153	390	488	Complete protection
	3	105	353	293	Protection with loss in weight in two cases. Scurvy in one case
30 g. cabbage steamed ½ hr. at 110° and white bread	3	96	393	327	Scurvy, with partial protection
30 g. cabbage steamed ½ hr. at 120°	3	45	387	268	All with severe scurvy

In contradistinction to their results, our experiments with a 15 g. ration of cabbage autoclaved at 110°–130° for one hour gave almost complete protection from scurvy in each case (see Table V). This difference in result is difficult to account for, unless it be due to the different technique, to the use of green rather than white cabbage or to heating without the addition of salt. The present experiments are in accordance with one another although they differ in results from those of the Norwegian investigators.

In order to get some basis for comparison with the experiments in which small rations of fresh cabbage were employed, we selected 5 g. as the standard ration for investigating temperatures below 100°, giving the animals (as in experiments with raw cabbage) an allowance of 60 cc. autoclaved milk daily. In many cases the animals voluntarily drank their milk, which was given morning and afternoon, but in cases of failing appetite through the onset of scurvy, or other disinclination, hand feeding with a glass syringe was adopted (cp. fig. 6, Plate I). As with the fresh cabbage experiments, only the outer green leaves were used for heating. We found it unnecessary to use salt in cooking. Most animals accepted the cooked cabbage readily and the rest were hand fed.

At 60°, 70° and 80° entire leaves were plunged into water contained in a French cooker surrounded by water at constant temperature. The temperature was kept under control and was observed at intervals during the time of cooking, and pieces weighing 5 g. were cut off after the cooking.

For the experiments at 90° and 100° heating was done in steam. Small pieces were cut from a fresh leaf, each weighing 5 g.; these were suspended singly from hooks on the inside of a saucepan lid which just fitted the steam chamber over a saucepan of boiling water. By adjusting the rate of boiling and also the steam outlet, the temperature of this chamber could be kept at 90° or 100° as required. In order to determine whether immersion in water at 100° itself caused any additional loss of antiscorbutic properties, a further set of animals were given a 5 g. ration taken from whole leaves which had been plunged into boiling water and boiled briskly for 20 minutes. They were then lightly drained, cut up and an amount equal to the 5 g. ration weighed out from them, an additional 0·5 g. being allowed to compensate for the extra water taken up in this method of cooking.

In all cases the cooking was done either daily or on alternate days, the cooked pieces being kept moist and placed in a refrigerator until wanted. The water content of the different samples was determined at intervals and varied from about 85–90 per cent., that of fresh cabbage varying from 87–90 per cent. of the fresh weight. The results of these experiments are summarised in Table IV and illustrated in the graphs, Fig. 3.

If the results are compared with those in Table II the following rough estimate of the degree of destruction of antiscorbutic material can be made. After one hour's heating at 60° the antiscurvy value of 5 g. cabbage is not greater than that of 1·5 raw cabbage, *i.e.* there is a loss of about 70 per cent. of the original value. Heating for the same time at 70° or 80° reduces the initial value of 5 g. raw cabbage to the equivalent of about 0·5 g. raw, *i.e.* a loss of 90 per cent. has taken place; after heating for one hour at 90° the loss exceeds 90 per cent.

From Table IV it is further evident that 5 g. cabbage heated either for one hour at 60° or for 20 minutes at 90°–100° has a similar effect in just protecting a young guinea-pig from scurvy. We have already seen that a daily ration of 1 g. fresh raw cabbage was the amount determined as necessary for protection from scorbutic symptoms (see p. 424). We may therefore regard 5 g. cabbage cooked for one hour at 60° as about equal in antiscorbutic value to 5 g. cabbage cooked at 100° for 20 minutes and to 1 g. raw cabbage. In other words the rate of destruction of antiscorbutic properties is increased about three times for a rise of temperature of 30° or 40°. If we assume that the destruction of antiscorbutic properties is uniformly accelerated by increase of temperature within this limit, the above results indicate the comparatively low temperature coefficient of about 1·3 for 10° rise of temperature. Assuming this value to be near the truth, then the effect of heating cabbage for one hour at 60° would be equivalent to heating for 46 minutes at 70°, or 35 minutes at 80°, or 28 minutes at 90°, or 21 minutes at 100°: and the experimental results show that the effect of heating 5 g. of cabbage for

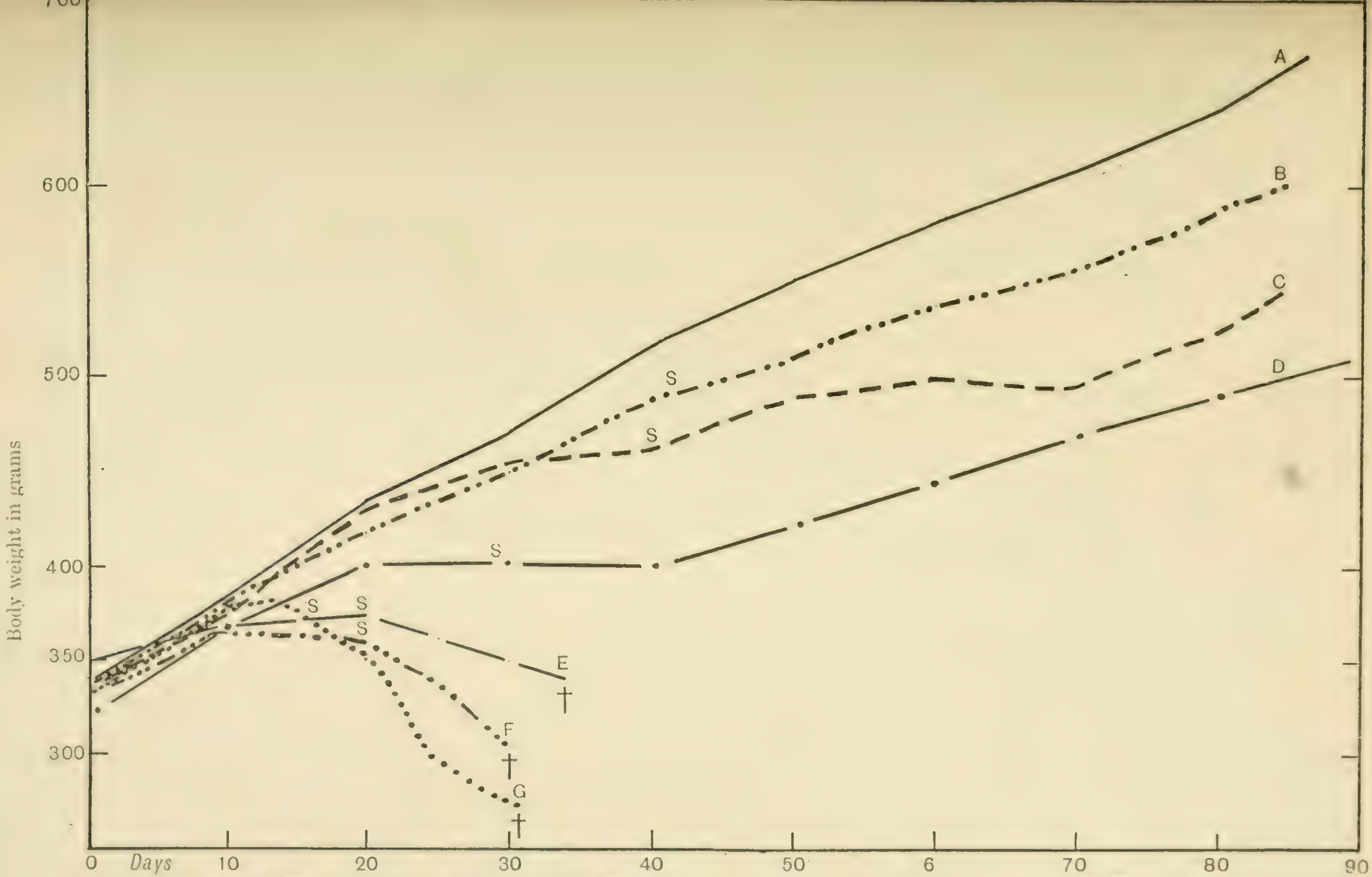

Fig. 3. Growth of young guinea-pigs upon a diet of fresh heated cabbage, oats, bran and autoclaved milk (Tables IV, VIII).

——— Curve A. Represents the standard growth of 3 animals upon 30 g. fresh cabbage, oats, bran and water (Expt. 1 Tables II and VII).

Curves B F. Represent each the average growth of 4 animals upon heated cabbage, oats, bran and autoclaved milk.

–••–•• Curve B. Cabbage heated to 60° for 1 hour (Expt. 8).

– – – – ,, C. ,, ,, 70 ,, ,, (Expt. 9).

–•••– ,, F. ,, ,, 90 ,, ,, At † an animal died and the average curve could not be continued (Expt. 11).

•••••• ,, G. Citric acid cabbage heated to 90° for 1 hour. At † an animal died and the average curve could not be continued (Expt. 11 A).

—•— Curve D. Cabbage heated to 100° for 20 mins. (Expt. 13).

—·— ,, E. ,, ,, 80 ,, 1 hour (Expt. 10).

At S symptoms first appeared in one or more animals.

one hour at 60° is, in fact, about equal to heating for 20 minutes at 90°–100° (see Table II).

This comparatively small influence of rise in temperature, between the temperatures 60° and 100°, upon the destruction of the antiscurvy value of cabbage is of some interest in connection with the unknown nature of the antiscorbutic factor. It has been suggested that this substance might be a complex protein or an enzyme-like substance. The rate of destruction of most proteins by heat is greatly accelerated with rise of temperature. Egg albumin is coagulated about 635 times faster at 70° than at 60° [Chick and Martin, 1910]; the destruction of emulsin by heat has a temperature coefficient of 7·4 for the same range of temperature [Tamman, 1895], and the action of trypsin is accelerated 5·3 times for every 10° rise in temperature [Bayliss, 1908]. The comparatively insignificant effect of temperature-rise in accelerating the gradual destruction of the antiscurvy substance within the tissues of cabbage leaves, indicates that heat denaturation of a protein-like or of an enzyme-like body is not primarily involved.

In the plant, the antiscorbutic substance is produced and retained in the living cell. On heating at 100° or higher temperatures, killing is almost instantaneous, the collapsed cells becoming permeable alike to water and to the constituents of cell sap. At lower temperatures the killing is more gradual (about 10–15 minutes at 60°) and the tissues remain tough and leathery even after an hour's heating at this temperature. These differences in the behaviour of the tissues on heating may affect the rate of destruction of the antiscorbutic substance. Similar experiments with expressed juices of vegetables and fruits are in progress and may be expected to throw light on this point. According to Holst and Frölich, expressed juice of white cabbage loses nearly all its protective value after heating for 10 minutes at 60°, 70°, or 80°, the expressed juice being, in their opinion, much more thermolabile than when contained in the intact cells. We are at present unable to confirm this statement and in preliminary experiments with expressed juice of turnips a considerable amount of protective value was found to exist after heating for an hour at 80°.

APPLICATION OF THE FOREGOING RESULTS TO METHODS OF COOKING VEGETABLES.

The destruction of the antiscorbutic properties of fresh vegetables on heating has an evident bearing on their dietetic value when cooked or preserved. Cooking improves most vegetables by softening their tissues and increasing

TABLE IV. *Experiments with 5 g. rations Cooked Cabbage (+ 60 cc. autoclaved milk).* [For further details see Table VIII.]

Exp.	Cabbage ration		No. of animals	Mean weight		Total gain in wt. %	Duration of exp. in days	Symptoms during life	Post-mortem	Histology of rib-junctions	General conclusions
	Temp. of heating	Time mins		Initial	Final						
8	60° immersed	60	4	339	601	71	88–90	Soreness after 42–60 days	3 normal 1 slightly scorbutic	2 normal 2 incipient scurvy	Protection
9	70 immersed	60	4	334	519	55	90	2 showed no symptoms, 2 developed soreness 42–88 days, 1 showing also "scurvy position"	1 normal 3 fragile bones, and rib-junctions ridged 1 with slight muscular haemorrhage also	2 nearly normal ("incipient scurvy") 2 acute scurvy	Protection Partial protection
10	80° immersed	60	4	347	—	—	34–50, 1 case 90	Soreness after 28–32 days Soreness acute at first, but lessening "scurvy position" in 2 cases	Fragile bones rib-junctions nodular muscular and subcutaneous haemorrhages } in each case	1 nearly normal ("incipient scurvy") 3 acute scurvy	Protection in 1 case, slight protection in 3 cases
11	90° steamed	60	4	334	—	—	21–50	Soreness after 19–35 days, scurvy position, loss in weight	Fragile bones, rib junctions nodular haemorrhages severe	All acute scurvy	No protection
11 A	90° steamed, (citric acid cabbage)	60	4	337	270	20	20–31	Soreness in 8–22 days	Acute symptoms	All acute scurvy	No protection
12	90° steamed	20	4	324	513	58	90	Soreness after 23–43 days No soreness in 1 case	All normal	All normal	Complete protection
13	100° steamed	20	4	329	485	17	90	3 with no symptoms 1 with slight persistent soreness after 27 days	do.	2 slight scurvy 2 normal	2 nearly protected 2 completely protected
14	100° immersed	20	4	327	493	50	3 lived for 90 (1 died in 30)	1 died in 30 days (pneumonia) with no symptoms; 1 lived 90 days—no symptoms; 2 developed soreness after 23 days	1 normal (after only 30 days) 3 with slight symptoms, brittle jaw, loose teeth, and in 1 case, ridged rib-junctions	1 normal 2 slight and 1 chronic scurvy	1 protected for 90 days 3 partially protected

their digestibility, but it certainly also causes some loss in their antiscorbutic value. This loss is the greater the longer the time of heating, and is very appreciable even when the temperature is far below the boiling point (*e.g.* 60°–70°). In general, slow cooking at a lower temperature is much more deleterious than more rapid cooking at a high one.

At the present time, owing to the shortage of fuel, there has been a great increase in the use of slow cooking of the hay-box type as a substitute for fire or gas cooking, both in private houses and in large communities, *e.g.* hospitals, factories, canteens, municipal kitchens. In this method of cooking, food is given 5–10 minutes' boiling on a stove, and then transferred to a closed box padded sufficiently to prevent much loss of heat by conduction. At least two hours is allowed in the hay box before the food is ready for consumption, and frequently four hours or longer is given, on the theory that in a hay-box it is impossible to over-cook the food. A well-made hay-box should only allow cooling to about 90° in two hours or to about 80° in four hours; but at these temperatures, as has been shown in the preceding section, the rate of destruction of the antiscorbutic factor is not much less than at 100°, although the softening of the tissues takes place much more slowly. It is clear therefore that the longer time needed in the hay-box is a great disadvantage in the case of green vegetables such as cabbage. So far as loss in antiscorbutic value is concerned, the effect of two hours in the hay-box is not much less than that of two hours' boiling.

In cooking green vegetables, it is customary to add a little soda to the water to preserve the green colour. It has been shown experimentally by other workers in this Institute [Harden and Zilva, 1918] that expressed orange juice loses its great antiscorbutic value when made even slightly alkaline shortly before administration. The destruction of the antiscurvy factor by weak alkalis would occur more rapidly at higher temperatures and would enhance the simple destructive effects of high temperature. At the present time, when vegetables form the chief source of antiscorbutic material in the diet of the great majority of the population, the use of soda in cooking should certainly be avoided.

The suggestion has been made[1], that, in order to lessen the inevitable loss of antiscorbutic properties suffered by vegetables on cooking, citric acid to the concentration of 0·5 per cent. should be added to the water in which they are boiled. This suggestion is based on the conclusion emphasised by Holst and Frölich, that fruit juices which are acid are more heat-stable in respect of the antiscorbutic factor, contained in them, than are the expressed juices of vegetables such as cabbage. According to these workers the juice of lemons and of raspberries retained its antiscurvy properties after heating to 100° for 60 minutes, whereas the freshly expressed juice of cabbage lost its antiscorbutic value after heating to 60°–100° for only 10 minutes. If however the expressed cabbage juice was acidified with 0·5 per cent. citric acid before heating some protection from scurvy was demonstrated. On the other hand expressed heated juice of dandelion leaves was no more protective when acidified, and sorrel leaf juice, which is acid in reaction when expressed, lost all its protective action when heated.

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[1] Private communication from Major E. D. W. Greig, I.M.S.

It therefore seemed worth while to investigate this point further and experiments were made on the influence of a weak solution of citric acid upon the antiscorbutic factor in cabbage leaves, both before and after heating. Four animals were fed on a 1·5 g. ration of raw cabbage leaves whose cut ends had 12–24 hours previously been placed in a 1 per cent. solution of citric acid; this passed readily up the vessels into the tissues of the leaf and could be demonstrated there with litmus paper. These four animals were given a ration of 60 cc. autoclaved milk daily and throve as well as those upon a 1·5 g. ration of raw cabbage without any citric acid (Exp. 6 A, Tables II and VII). When an acidified ration of 5 g. was heated to 90° for an hour, however, it had no longer any protective value. Four animals kept on a diet of oats and bran, 60 cc. autoclaved milk and 5 g. acidified heated cabbage died of acute scurvy in 22–30 days. Untreated cabbage heated for this length of time at 90° gave very slight protection, four animals on this ration dying of scurvy in 21–50 days. The addition of the acid therefore had certainly not improved the resistance of the cabbage to the destructive effects of heat[1].

The conclusion therefore to be drawn from these results is that in cooking vegetables it is better to add neither acid nor alkali to the water in which they are boiled.

INFLUENCE OF EXPOSURE TO TEMPERATURES ABOVE 100° UPON THE ANTISCORBUTIC VALUE OF CABBAGE.

In order to get evidence as to the effect of heating at higher temperatures it was necessary to use larger rations. With an allowance of 15–30 g. daily, satisfactory nutrition was obtained, without the introduction of autoclaved milk into the diet, even when the cabbage had been heated to 100°–130° for one hour. For example, growth curves of animals fed daily on 15 g. cabbage steamed at 100° for an hour compared favourably with the growth curves of similar animals fed on 15 g. raw cabbage (cf. curve C, fig. 4, with B, fig. 1). Two animals were paired after 60 days of this diet and produced healthy young while still upon this limited cabbage ration.

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[1] This result has been confirmed in case of germinated lentils; when boiled for 15 minutes in water containing 0·5 per cent. citric acid, the destruction of antiscorbutic properties was found to be distinctly greater than when boiled for the same period in pure water.

A summary of the results obtained on heating cabbage to temperatures from 100°–130° as regards loss of antiscorbutic properties is set out in Table V and full protocols of the individual experiments may be found in Table VIII. Curves showing the average growth are shown in Figs. 4 and 5. It will be seen that protection from scurvy was obtained with the 15 g. ration in all cases. Even after heating to 130° for an hour, the symptoms of scurvy in life were only slight and at post-mortem examination the signs consisted of slight ridges upon the rib-junctions in three cases out of four, and small muscle haemorrhages in two cases out of four. All animals in Exps. 15–21, Table V, were in good health after three months upon the specified diets. If, however, slighter manifestations of scurvy were carefully looked for, there is on the whole progressive increase in the degree to which these are apparent, as the temperature at which the rations are heated is raised from 100°–130°. This is indicated by the increase of the number showing slight abnormalities of the histology of the rib-junctions and by gradations from slight tenderness (Expts. 18, 19, cabbage heated at 110° and 120° respectively for one hour) to definite and permanent soreness and swelling of the joints (Expts. 21 and 22, where the cabbage was heated for one and for two hours respectively at 130°).

In Exp. 21 (cabbage heated at 130° for one hour) slight but definite muscular haemorrhages were found beneath the muscles of the knee in two out of four animals after 90 days upon this diet. But not even in this case can the animals be said to have developed well-marked scurvy, and it is clear that there is a large margin of protection against scurvy in the raw 15 g. ration. After heating to 130° for one hour this becomes reduced to the equivalent of rather less than 1·5 g. raw cabbage and greater than 0·5 g. raw cabbage (as may be deduced from a comparison of symptoms with those of Expts. 6 and 7, Table II), and may be estimated at approximately the equivalent of 1 g. raw cabbage. On this basis we arrive at the conclusion that on heating for one hour at 130°, 15 g. of cabbage loses $\frac{14}{15}$, or 93 per cent., of its original antiscurvy properties. In Exp. 19, cabbage heated for one hour at 120°, the five animals were protected from any marked symptoms of scurvy, and the residual antiscorbutic factor may be estimated as, at least, the equi-

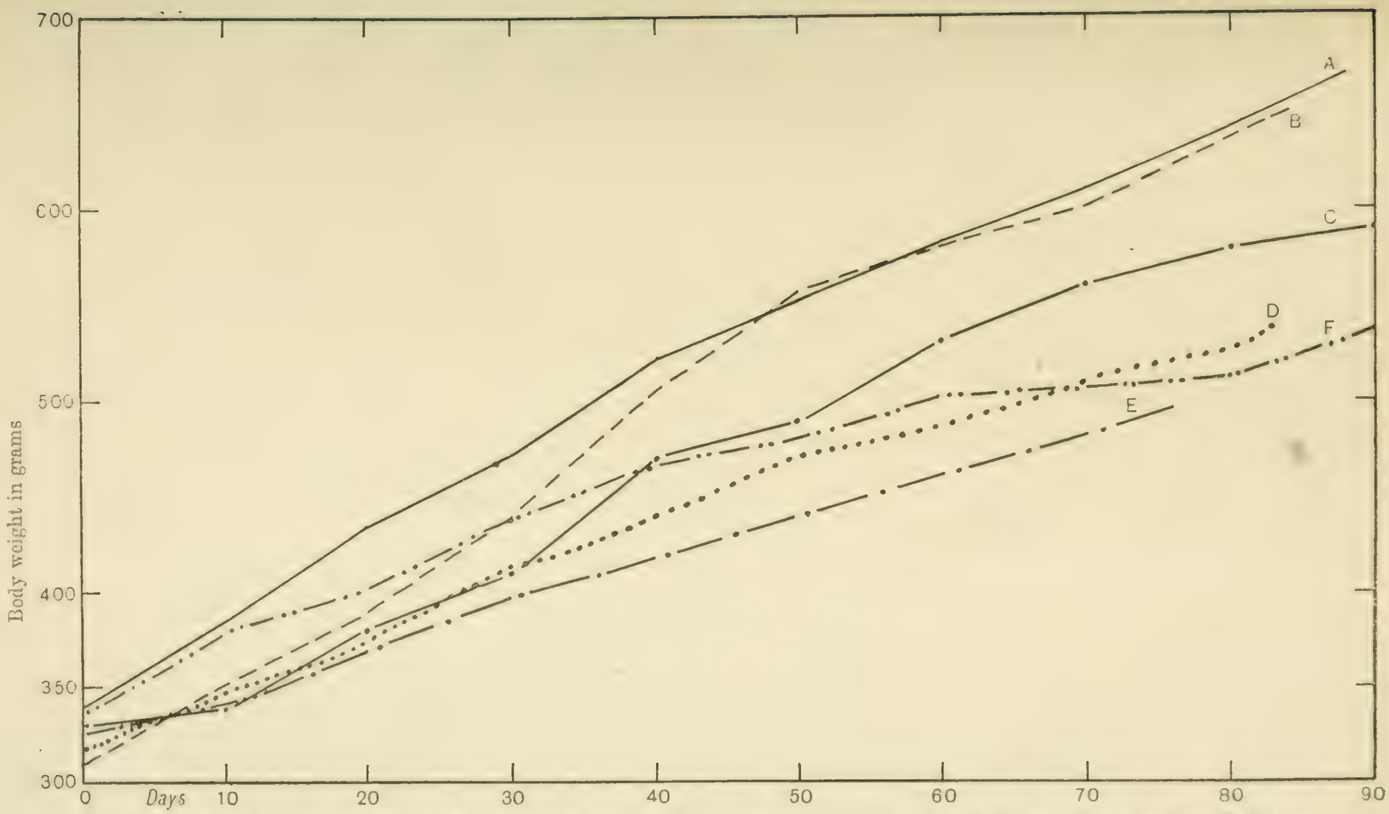

Fig. 1. Mean weight curves of young guinea-pigs on a diet of cabbage cooked 1 hour, oats and bran *ad lib.* and water. Each curve represents the mean weight of all the animals in one experiment calculated from time to time (Tables V and IX).

——— Curve A. Represents the standard growth curve of 3 animals on a 30 g. ration of raw cabbage (Expt. 1, Tables II and VII).
– – – ,, B. Represents the mean growth curve of 3 animals on a 30 g. ration of cabbage cooked at 100° for 1 hour (Expt. 15).
—•— ,, C. ,, ,, ,, 4 ,, 15 ,, ,, 100 ,, (Expt. 16).
•••••• ,, D. ,, ,, ,, 4 ,, 15 ,, ,, 110 ,, (Expt. 18).
•—•—• ,, E. ,, ,, ,, 4 ,, 15 ,, ,, 120 ,, (Expt. 19).
—••—•• ,, F. ,, ,, ,, 4 ,, 15 ,, ,, 130 ,, (Expt. 21).

TABLE V. *Experiments with* 15 *g. cabbage + oats and bran + water.* [For details see Table IX.]

Exp.	Ration	Number of animals	Mean initial weight in g.	Total gain %	Time of exp. in days	Symptoms in life	Post-mortem examination	Histology of rib-junctions	General conclusions
15	30 g. cabbage steamed 1 hr. 100	3	317	109	90	None	Normal	Normal	Complete protection
16	15 ,, ,, ,, 1 ,, ,,	3	327	82	90	do.	do.	do.	do. do.
17	15 ,, ,, ,, 2 ,, ,,	5	337	56	90	Temporary soreness in 1 case	Normal (fragile bones in 1 case)	do.	do. do.
18	15 ,, ,, ,, 1 ,, 110°	5	318	71	85–112	Slight soreness	Normal	Nearly normal or normal	Complete protection (nearly complete protection in 1 case)
19	15 ,, ,, ,, 1 ,, 120°	4	326	56	90	Sore knees in 1 case	Jaw fragile in 3 cases	Normal in 2 cases, nearly normal in 2 cases (incipient scurvy)	Complete protection in 2 cases, protection in 2 cases
20	15 ,, ,, ,, 2 ,, 120°	4	330	70	90	Soreness in all after 18–26 dys	Bones slightly brittle	3 normal 1 nearly normal	Complete protection in 3 cases, protection in 1 case
21	15 ,, ,, ,, 1 ,, 130°	4	346	55	90	Soreness in all after 15–28 dys	Nearly normal, slight haemorrhages in 2 cases	2 normal 2 incipient scurvy	2 complete protection 2 protection
22	15 ,, ,, ,, 2 ,, 130°	3	343	12	88	Sore knees in 32 days	Bones fragile, teeth loose	2 definite scurvy 2 incipient scurvy	2 partial protection 2 protection
	(with milk)	1	335	100	86				

valent of 1·5 g. raw cabbage, *i.e.* a loss has taken place not exceeding 90 per cent. When heating was continued for a second hour at this temperature the antiscorbutic value was still not reduced below that required for protection. On heating for two hours at 130°, however, definite scurvy symptoms in the form of swollen joints were apparent within 40 days. In this case the 15 g. ration of heated cabbage may be reckoned equivalent to something less than 1 g. but distinctly more than 0·5 g. raw cabbage in antiscurvy value and the loss suffered in this respect may be estimated at more than 93 per cent., and less than 97 per cent.

These rough estimates are collected in Table VI, and for purposes of comparison, the results of the preceding experiments in which the heating was done at temperatures between 60° and 100° are included. It is surprising to find that the destruction at the higher temperatures (Exps. 16–22) is not more complete. Even with the larger ration of 15 g., if the proportional destruction of antiscorbutic factor had proceeded on the same lines as at the temperatures below 100°, we should expect to find the residual amount reduced below the minimum required for protection (the equivalent of about 1 g. raw cabbage) at a temperature much lower than 130°, with one hour's heating. As a matter of fact, definite scorbutic symptoms occurred only when the *15 g. ration was heated for two hours at 130°.*

Table VI. *Showing approximately the degree of destruction sustained by the antiscorbutic and growth-promoting properties respectively, of fresh cabbage leaves when exposed to various temperatures.*

				Antiscurvy value		Growth promoting value	
Exp. no	Ration of cabbage, g.	Time of heating, minutes	Temperature C.°	Approximate estimate of heated material expressed in g. of raw cabbage	Loss expressed as % of the original	Approximate estimate of heated material expressed in g. of raw cabbage	Loss expressed as % of the original
8	5	60	60	1·5	70	—	—
9	5	60	70	0·5	90	—	—
10	5	60	80				
11	5	20	90	1·5	70	—	—
12	5	60	90	less than 0·5	more than 90	—	—
13	5	20	100	1·5	70	—	—
16	15	60	100	more than 1·5	less than 90	15	0
17	15	120	100	do.	do.	greater than 5	less than 66
18	15	60	110	do.	do.	—	—
19	15	60	120	do.	do.	—	—
20	15	120	120	more than 1	less than 93	—	—
21	15	60	130	1	93	5	66
22	15	120	130	less than 1 greater than 0·5	more than 93 less than 97	0	100

This point is shown perhaps even better by comparing the experiments in which the heating was done at 100° for both larger and smaller ration. Taking Exp. 13 as standard, with a loss of approximately 70 per cent. antiscurvy value after 20 minutes' heating at 100°, and applying this result to Exp. 16, we should expect the antiscurvy factor of the 15 g. ration heated for one hour at 100° to be reduced to the equivalent of 4·5 g. raw cabbage at the end of 20 minutes: to that of 1·4 g. raw cabbage at the end of 40 minutes: and to that of 0·42 g. raw cabbage at the end of 60 minutes. In other words the animals in Exp. 16 should have developed scurvy to a degree similar to that shown by the animals upon a 0·5 g. ration of raw cabbage, see Exp. 7, Table II. As a matter of fact they did nothing of the sort, but remained in excellent health throughout and were enabled to breed satisfactorily upon the diet.

It is therefore not possible to apply, by extrapolation, the results of experiments with a 5 g. cabbage ration to those in which a 15 g. ration is heated at higher temperature or for a longer time. There is no obvious explanation for this discrepancy, and we must conclude that in diets of this type, the *size* of the cabbage ration has some influence upon the degree to which its antiscurvy properties are destroyed by heat. It is evident that with the larger ration some extra source of protection from scurvy is being made available. In searching for this source, another point of difference in the two sets of experiments, is that the source of the fat-soluble growth factor is also different in the two cases. In case of the 5 g. ration, it is mainly derived from the allowance of autoclaved milk; in case of the 15 g. ration, it is derived wholly from the cabbage ration. It is not at once evident in what way this circumstance can affect the antiscorbutic reserves of the diet, and there is much evidence accumulated by the group of workers in this Institute, pointing to a distinct and separate action of these two factors in the nutrition of young guinea-pigs. Miss Tozer has, however, found that a lack of fat-soluble growth factor in the diet is accompanied by a series of changes in the histology of the rib-junction which is almost indistinguishable from that caused by a slight deficiency of antiscorbutic factor (see below, Appendix). It is therefore not impossible, that the antiscorbutic value of a diet may be enhanced and may show greater heat stability, when the antiscurvy factor and the growth fat-soluble factor are derived from the same food stuff. It is noteworthy that scorbutic symptoms supervened in animals fed with the 15 g. cabbage ration which had been heated to 130° for two hours, a point at which, as will be seen below, the reserves of the fat-soluble growth factor were also exhausted. This factor is believed to be comparatively heat stable and the present investigation affords confirmatory evidence upon this point.

The suggestions contained in the preceding paragraph are put forward with reserve, especially as experiments are now in progress from which it is hoped that more light may be obtained. An investigation is being made on the influence of heat upon the antiscorbutic properties of expressed fruit and vegetable juices, which are deficient in the fat-soluble growth factor. This is supplied by a ration of autoclaved milk, and as the temperature of heating is raised the only change in the diet will be in the allowance of antiscorbutic material provided, the amount and source of the fat-soluble growth factor remaining unchanged throughout. This further investigation should also show whether the inclusion of the antiscorbutic factor in a living tissue has any influence upon its heat stability and the discussion of the whole subject may be profitably postponed until it is completed.

INFLUENCE OF HEAT UPON THE GROWTH-PROMOTING PROPERTIES OF CABBAGE.

It has been shown that for full normal growth in a young guinea-pig on a cereal diet, a large daily ration of fresh cabbage (about 30 g.) is necessary. When this is reduced to 15 g. or further to 5 g. daily the resulting growth is also correspondingly diminished (see Table II and Fig. 1). There was no marked difference in appetite amongst the animals of the different experiments for the cereal part of the diet (Table VII), and the diminished growth can best be explained as caused by the lack of sufficient amount of some accessory growth factor in the diet. The work of McCollum and his colleagues, and of Osborne and Mendel has demonstrated that in the case of rats, two accessory growth factors are necessary in the diet if growth is to be made. In our experiments the water soluble growth factor was provided abundantly in the oats and bran ration *ad libitum* [McCollum and Davis, 1915, 1], whilst the fat-soluble growth factor shown to be widely distributed in green leaves [McCollum, Simmonds and Pitz, 1916] was strictly limited. It seems therefore that the limitation of the amount of growth on reducing the cabbage ration is due to the lack of fat-soluble A in the diet. This is in agreement with the general statement of McCollum and Davis that a small amount of the accessory substances is necessary for growth and that "above this amount growth appears to be in some measure proportional to the amount of the accessories present" [McCollum and Davis, 1915, 1]. When the 60 cc. ration of autoclaved milk was added to the diets containing the small cabbage rations, this deficiency was repaired.

In the results of Expts. 3 and 4, Table II, a diet of 5 g. fresh cabbage with oats and bran and water can be compared with a similar diet to which 60 cc. daily of milk previously autoclaved at 120° for one hour has been added (cp. Fig. 1, curve *C*, and Fig. 2, curve *B*). The animals on the diet which included milk made much better growth than those without it, and their average weight-increase nearly equalled that of animals receiving 15 g. fresh raw cabbage daily and no milk. As regards growth, therefore, cabbage and autoclaved milk can replace each other. In the case of smaller rations of cabbage, *i.e.* of 2·5, 1·5 and especially of 0·5 g. cabbage, in spite of the consumption of the daily ration of autoclaved milk, growth never equalled that upon a 15 g. ration (Fig. 2). In these cases it appears that the reduction of the anti-scorbutic factor may itself have limited growth.

Both growth factors have been regarded as comparatively heat stable. Osborne and Mendel [1915] have shown that the growth promoting powers of butter fat remain unimpaired after heating for two hours at 100°. Other workers in this Institute have drawn attention to the growth promoting effects of milk which has been heated in an autoclave for an hour at 120° [Chick, Hume and Skelton, 1918]. More recently, Steenbock, Boutwell and Kent [1918] working with rats have demonstrated a destruction of the fat-soluble growth factor in butter fat after heating for four hours at 100°. These authors consider that the destructive process is one of low velocity which had not been revealed in the work of other investigators owing to the high initial content of the factor in the original material. These observations do not give any direct evidence as to the rate of destruction of the growth promoting substance, since no systematic comparison of the effects of heated rations has been made with a minimal raw standard ration.

By studying the growth curves of the animals fed on 15 g. cabbage rations heated at various temperatures, it is possible to investigate the influence of temperature on the fat-soluble growth factor.

Comparing C, fig. 4, and B, fig. 1, it is clear that the effect of heating a 15 g. ration of cabbage for one hour at 100° is not accompanied by any change in the amount of growth which was made on this diet, although a 15 g. raw cabbage ration is itself limiting, in that it does not allow maximum growth. A comparison of the slopes of the graphs of Fig. 4 shows that there is a progressive slight decrease in growth rate (10–20 per cent.) as the temperature of heating is raised from 100° to 130°. This can best be explained as the result

TABLE VII. *Experiments with raw cabbage rations.*

Exp.	No.	Sex	Ration of cabbage, g.	Wt of animal, g.		Gain % on total weights	Time of exp. in days	Symptoms	Post mortem	Histology of rib junctions	Food consumed			General conclusions
				Initial	Final						Oats, bran, g.	Cabbage, g.	Aut. milk, cc.	
1	351	♂	30 + water	350	670	91	87	No symptoms	Not examined	Not examined	57	30	—	Complete protection
	352	♀		340	717		92	do.	do.	do.	47	29	—	do.
	865	♀		330	675		90	do.	do.	do.	41	21	—	do.
	885	♂		340	615		83	do.	do.	do.	61	30	—	do.
2	353	♀	15 + water	330	555	71	90	do.	do.	do.	40	15	—	do.
	359	♀		350	580		90	do.	do.	do.	59	15	—	do.
	826	♀		330	620		90	do.	do.	do.	53	15	—	do.
	823	♀		370	650		90	do.	Normal	Normal	55	15	—	do.
3	121	♂	5 + water	330	525	26	90	Soreness after 37 days, persistent	Slight haemorrhages; bones slightly brittle	Nearly normal (incipient scurvy)	55	5	—	Protection
	122			320	445		90	No soreness	Normal	do. do.	61	5	—	do.
	125	♂		318	274		90	do.	do.	do. do.	39	5	—	do.
4	120	♀	5 + aut. milk	340	530	69	90	Soreness after 44 days, persistent	Slight haemorrhages at knee	do. do.	42	5	55	do.
	123	♀		300	550		90	No symptoms	do. do	do. do.	26	5	57	do.
	124	♀		335	565		90	Soreness after 24 days, persistent	Normal	do. do.	39	5	52	do.
5	140	♀	2·5 + aut. milk	320	530	49	90	No symptoms	do.	do. do.	60	2·5	55	do.
	141	♀		302	430		90	Soreness after 26 days, lasting 20 days	do.	do. do.	35	2·5	42	do.
	143	♀		250	440		88	No symptoms	Tibia brittle	do. do.	35	2·5	55	do.
	379	♀		340	515		90	Soreness after 40 days, persistent	Normal	do. do.	56	2·5	45	do.
	380	♂		305	498		90	No symptoms	do.	do. do.	54	2·5	46	do.
6	389	♀	1·5 + aut. milk	365	502	49	90	Soreness after 35 days, lasting 23 days	do.	Normal	34	1·5	53	do.
	390	♀		330	478		90	No symptoms	do.	do.	48	1·5	45	do.
	391	♂		320	350		70	Loss of weight, no soreness	do.	Slight scurvy	38	1·5	49	do.
	392	♀		355	520		90	Soreness after 38 days, persistent	Jaw fragile	Normal	45	1·5	53	Slight scurvy
	861	♂		355	675		90	Soreness after 33 days, persistent	Normal	do.	46	1·5	59	Protection
	862	♀		325	404		90	Soreness after 87 days, persistent	Femur and tibia brittle; ribs ridged; jaw fragile	Nearly normal (incipient scurvy)	21	1·5	52	do.
6 A	831	♀	1·5 (citric acid) + aut. milk	325	538	79	91	Soreness after 50 days, lasting only 10 days	Normal	Nearly normal (incipient scurvy)	69	1·5	60	do.
	832	♀		372	535		90	Soreness after 50 days, persistent	Jaw fragile, slight haemorrhages	do. do.	28	1·5	60	do.
	833	♂		300	615		90	Soreness after 49 days, lasting 34 days	Normal	Normal	63	1·5	60	Complete protection
	834	♂		322	565		90	Soreness, doubtful throughout	Rib junctions slightly ridged	Nearly normal	34	1·5	59	Protection
7	840	♂	0·5 + aut. milk	335	427	18	90	Soreness after 79 days, persistent	Tibia brittle; 5 ribs ridged; slight haemorrhages	Normal	28	0·5	60	do.
	841			330	403		90	Soreness after 30 days, persistent Scurvy position	Tibia and femur brittle; 7 ribs ridged; teeth brittle	Chronic scurvy	25	0·5	58	Chronic scurvy
	847			328	275		67	Swollen knees; loss in weight	Tibia and femur fragile; all ribs ridged; jaw fragile; haemorrhages	Acute scurvy	15	0·5	38	Acute scurvy
	848			330	460		90	Soreness and swollen knees after 26 days, persistent	Four ribs ridged	Slight scurvy	21	0·5	69	Slight scurvy

Exp.	Ration of cabbage, g.	Time of heating, mins.	Temperature °C.	No. of animal	Mean weight of animals, g.		Gain in wt %	Time of exp. in days	Symptoms during life	Results of post-mortem examination	Results of histological examination	Food consumed, g.			General result
					Initial	Final						Oats, bran	Aut. milk	Cabbage	
8	5	60	60	866 ♂	355	615	71	86	Soreness after 42 dys	Normal	Nearly normal (incipient scurvy)	24	59	5	Protection
				867 ♀	320	568		90	,, ,, 42 ,,	3 rib-junctions ridged, lower molars brittle	do. do.	27	59	5	do.
				868 ♀	320	665		90	,, ,, 60 ,,	Normal	Normal	29	60	5	do.
				869 ♀	360	557		90	,, ,, 43 ,,	do.	do.	34	60	5	do.
9	5	60	70	835 ♀	325	437	55	91	No symptoms	do.	Nearly normal (incipient scurvy)	44	58	5	do.
				836 ♂	322	428		64	do.	Tibia fragile	do. do.	24	58	5	Protection (cause of death unknown)
				837 ♂	360	790		90	Soreness after 42 dys, scurvy position	3 rib-junctions ridged	Acute scurvy	43	60	5	Definite scurvy
				838 ♀	330	420		91	Soreness after 88 dys	Tibia and jaw fragile; 5 rib-junctions ridged; slight muscular haemorrhages	do.	25	56	5	do.
10	5	60	80	805 ♂	345	395	−8	89	Soreness after 32 dys, with partial recovery	Jaw and teeth brittle; 5 rib-junctions ridged; haemorrhages in muscles	Nearly normal (incipient scurvy)	47	52	5	Partial protection (recovery from scurvy)
				806 ♀	355	356		34	Soreness after 32 dys; scurvy position	All bones very fragile; severe muscular haemorrhages	Acute scurvy	24	52	5	No protection. Death from acute scurvy
				807 ♀	335	270		50	Soreness after 28 dys; scurvy position	do. do.	do.	41	41	5	Slight protection. Death from acute scurvy
				808 ♂	355	263		50	Soreness after 29 dys; scurvy position	do. do.	Definite scurvy	35	44	5	Slight protection. Death from acute scurvy
11	5	60	90 (steamed)	381 ♀	335	240	−19	29	Soreness after 27 dys	do. do.	Acute scurvy	22	41	5	Death from acute scurvy
				382 ♂	350	280		49	,, ,, 35 ,,	do. do.	do.	21	52	5	do. do.
				383 ♂	330	275		29	,, ,, 21 ,,	do. do.	do.	20	34	5	do. do.
				384 ♂	320	285		21	,, ,, 19 ,,	do. do.	do.	22	30	5	do. do.
11A	5 (citric acid)	60	90 (steamed)	393 ♀	330	255	−14	26	,, ,, 17 ,,	do. do.	do.	26 (17 dys)	52	4·9 (22 dys)	do. do.
				394 ♂	355	270		31	,, ,, 22 ,,	do. do.	do.	29	46	4·8	do. do.
				395 ♂	340	305		30	,, ,, 21 ,,	do. do.	do.	25	50	5	do. do.
				396 ♂	325	250		26	,, ,, 8 ,,	do. do.	do.	31	51	5	do. do.
12	5	20	90 (steamed)	801	340	510	58	90	Slight soreness after 30 dys, persistent	Normal	Normal	55	53	5	Complete protection
				802	320	458		90	Slight soreness after 43 dys, persistent	Nearly normal (jaw fragile)	do.	28	54	5	Protection
				803	320	575		90	Slight soreness after 23 dys (temporary)	Normal	do.	34	59	5	Complete protection
				804	315	508		90	No symptoms	do.	do.	36	58	5	do.
13	5	20	100 (steamed)	397 ♀	325	342	48	90	do.	do.	Definite scurvy (chronic)	35	57	5	Partial protection (chronic scurvy)
				398 ♀	338	520		90	do.	do.	Normal	67	58	5	Complete protection
				399 ♀	330	530		90	do.	do.	Definite scurvy (chronic)	27	49	5	Partial protection (chronic scurvy)
				399a ♂	325	560		90	Slight soreness after 27 dys, persistent	do.	Normal	41	56	5	Protection
14	5	20	100 (immersed)	385 ♀	330	238	31	28	No symptoms	Emaciated; muscular and subcutaneous haemorrhages	Definite scurvy	33	30	5	Partial protection (died of pneumonia)
				386 ♂	330	578		90	do.	Jaw fragile; 2 rib-junctions ridged	Nearly normal (incipient scurvy)	49	55	7	Protection
				387 ♀	320	360		90	Soreness after 23 dys, acute, but not persistent	Jaw fragile; teeth loose	Acute scurvy (chronic)	29	55	5	Protection (chronic scurvy)
				388 ♀	332	540		90	Soreness after 23 dys	Jaw fragile	Normal	42	46	5	Protection

TABLE IX. *Experiments with 15 g. heated cabbage rations.*

Cabbage ration, g.	Heated		Animal		Wt of animals, g.		Duration of exp. in days	Mean gain %	Symptoms	Post-mortem	Histology of rib-junctions	Food eaten, g.		General conclusion
	Temp. °C.	Time, hours	No.	Sex	Initial	Final						Oats, bran	Cabbage	
30	100	1	333	♂	330	760	84	109	No symptoms	Normal	Normal	41	30	Complete protection
			334	♂	305	620	84		do.	Not examined	Not examined	47	30	,, ,,
			335	♂	315	610	84		do.	do.	do.	37	25	,, ,,
15	100	1	338	♂	300	598	90	82	do.	Normal	Normal	45	15	,, ,,
			339	♀	330	540	90		do.	Not examined	Not examined	40	15	Produced 1 young after experiment
			340	♂	350	645	90		do.	do.	do.	44	15	
15	100	2	354	♂	330	553	90	56	Soreness, temporarily, after 36 days' experiment	Normal	Normal	45	15	Complete protection
			355	♀	330	505	90		No symptoms	Not examined	Not examined	32	15	Two young; one born dead -
			356	♂	355	465	90		do.	Muscles dark in colour	Normal	41	15	Protection
			357	♂	340	530	90		do.	Tibiae brittle; jaw fragile	do.	39	15	,,
			358	♂	330	560	90		do.	Jaw fragile	do.	35	15	,,
15	110	1	365	♂	325	602	90	71	Temporary soreness for 3 wks after 24th day	Not examined	Not examined	41	15	,,
			366	♂	337	660	90		No symptoms	do.	do.	43	15	,,
			367	♀	308	455	86		Soreness, persistent, after 63 dys	Normal	Normal	36	15	,,
			368	♂	296	505	85-112		Soreness, persistent, after 70 dys	do.	do.	46	15	,,
			369	♀	325	498	90		No soreness	Not examined	Not examined	50	15	,,
15	120	1	374	♀	315	510	90	56 (excluding No. 378)	Soreness after 56 dys, persistent	Muscles flushed	Nearly normal (incipient scurvy)	45	15	,,
			375	♂	345	517	90		No symptoms	Normal	Normal	37	15	,,
			376	♂	308	507	90		do.	Jaw fragile	do.	47	15	,,
			377	♂	335	515	90		do.	Not examined	Not examined	46	15	,,
			378	♂	335	456	46		Soreness after 16 dys, persistent	Slight muscular haemorrhage	Normal	37	15	,,
15	120	2	853	♂	325	622	90	70	,, ,, 33 ,, ,,	Normal	do.	41	15	,,
			854	♀	322	532	87		,, ,, 18 ,, ,,	Two rib-junctions ridged; severe visceral haemorrhage	do.	41	15	,,
			855	♀	325	310	35		Died of diarrhoea	Jaw and tibiae brittle	Nearly normal (incipient scurvy)	28	15	,,
			856	♀	345	515	?		Soreness after 35 dys, persistent	Slight haemorrhages beneath each knee	Normal	44	15	,,
			870	♀	330	575	89		,, ,, 56 ,, ,,		do.	44	15	,,
15	130	1	881	♂	349	656	91	55	,, ,, 15 ,, ,, scurvy position	Slight haemorrhages beneath each knee	Normal	47	15	,,
			882	♂	330	515	90		Soreness after 16 dys, persistent, 64 dys, faceache position	5 rib-junctions slightly ridged	do.	38	15	,,
			883	♀	348	522	91		Soreness after 27 dys, persistent	Slight haemorrhages beneath knees. All rib-junctions ridged, 3 with nodules	Nearly normal (incipient scurvy)	44	15	,,
			884		358	450	89		Soreness after 28 dys, persistent, but improving later	Rib-junctions narrowly ridged; molars loose	do. do.	29	15	,,
15	130	2	911	♀	330	315	90	12	Soreness after 31 dys, swollen knees	Tibiae brittle, ribs papery, jaw and teeth brittle	Definite scurvy	27	15	Partial protection
			912	♂	350	395	88		Soreness after 31 dys, slightly swollen knees	Ribs ridged, teeth brittle and loose	,, ,,	28	15	,, ,,
			913	♀	350	413	88		Soreness after 20 dys, right knee sore, becoming swollen	Slight haemorrhages	Incipient scurvy	30	15	Protection

of the destruction of some part of the fat-soluble factor during heating, seeing that the animals were otherwise in good health and there were no definite symptoms of scurvy. This destruction is increased with increase of time (Fig. 5) and at 130° there is a sharp contrast between the mean growth curve of animals fed on the ration heated for one and two hours respectively, the animals on the ration heated for two hours making practically no growth, although remaining very active. One of these animals was given a ration of 60 cc. autoclaved milk daily after the 30th day, and its weight at once rose 66 g. in the next 14 days, and was doubled by the end of the experiment (Table IX), showing that the increase of the fat-soluble factor in the diet caused an immediate access of growth.

By comparing the slopes of the average curves (Figs. 1–4) some idea of the rate of destruction of the fat-soluble growth factor in cabbage leaves at 100°–130° may be obtained. Curves representing the mean growth of experimental animals kept for 90 days on a 15 g. ration given raw (B, Fig. 1) and heated one hour at 100° (C, Fig. 4) are nearly identical. Cooking for an hour has therefore left the growth promoting powers but little impaired. When heating is continued at this temperature for two hours, the resulting growth is much less than that on a 15 g. raw ration but greater than that on a 5 g. raw ration in absence of milk, *i.e.* about half the original amount of growth factor has apparently been destroyed. The mean growth on a ration of 15 g. cabbage heated to 130° for one hour is approximately the same as that of animals on a 5 g. raw cabbage ration; this would indicate a destruction of 60–70 per cent. of the growth factor originally present. After two hours' heating at 130° little or no accessory growth factor remains. On the whole therefore, the fat-soluble growth factor is less sensitive to heat than the antiscorbutic factor. These conclusions are summarised in Table VI.

SUMMARY AND GENERAL CONCLUSIONS.

1. It has been shown experimentally that a very small amount of raw cabbage daily is sufficient to protect from symptoms of scurvy, when added to a diet of grain and autoclaved milk. Histological evidence shows, however, that an apparently healthy animal may be in a condition of incipient scurvy as regards the structure of its growing bones. Possibly these animals might develop symptoms of the disease if kept under less favourable conditions.

2. By a similar method of experiment, determination has been made of the antiscurvy value of cabbage after exposure to various temperatures for

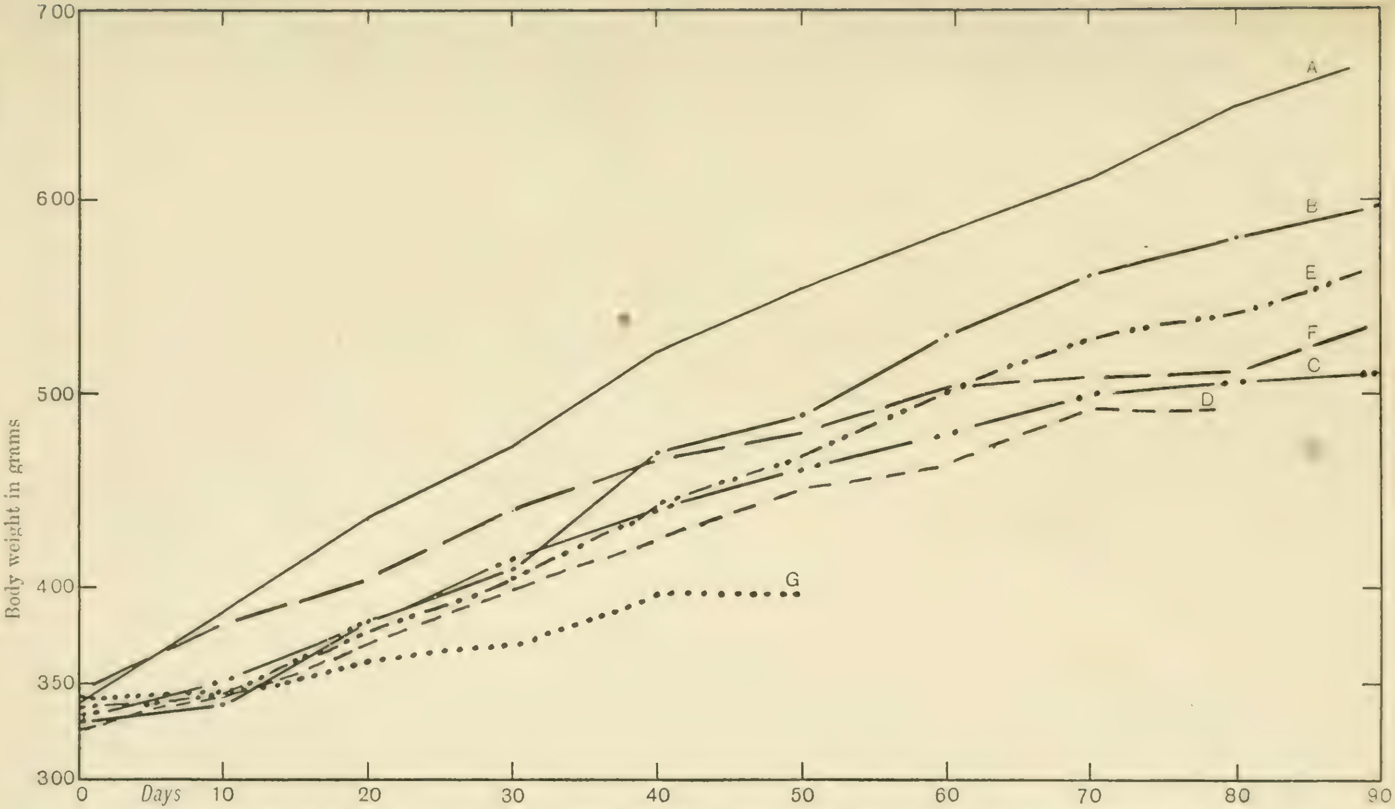

Fig. 5. Comparison of growth of animals fed on oats, bran and water (Tables V and IX), and on 15 g. cabbage heated at 100–130° for varying lengths of time.

——— Curve A. Represents the standard growth on 30 g. fresh raw cabbage, oats, bran and water (Expt. 1, Tables II and VII).
—·— ,, B. Represents the average growth of 4 animals on cabbage heated at 100° for 1 hour (Expt. 16).
—●— ,, C. ,, ,, ,, 4 ,, ,, ,, ,, 100° ,, 2 hrs. (,, 17).
– – – ,, D. ,, ,, ,, 4 ,, ,, ,, ,, 120° ,, 1 hour (,, 19).
-·-·- ,, E. ,, ,, ,, 4 ,, ,, ,, ,, 120 ,, 2 hrs. (,, 20).
— — ,, F. ,, ,, ,, 4 ,, ,, ,, ,, 130° ,, 1 hour (,, 21).
••••••• ,, G. ,, ,, ,, 3 ,, ,, ,, ,, 130° ,, 2 hrs. (,, 22).

different intervals of time, and comparison made with that determined for raw cabbage. The antiscurvy factor was found to be exceedingly sensitive to temperatures below 100°. It is estimated that about 70 per cent. of the original value is lost after one hour's heating at 60° and over 90 per cent. after the same period at 90°. After 20 minutes' heating at 90° or 100°, the loss was estimated at about 70 per cent., *i.e.* the same as after 60 minutes at 60°.

3. It follows from the results detailed under (2) that the rate of destruction of the antiscorbutic accessory factor in cabbage leaves is increased only about threefold for an increase in temperature of 30°–40°. This low temperature coefficient is in opposition to the view that heat-denaturation of a protein-like or enzyme-like body is here in question.

4. These facts have some bearing on methods of cooking green vegetables, and indicate broadly that the least loss of antiscorbutic properties will be obtained by cooking green vegetables for a short time at a higher temperature rather than for a longer time at a lower temperature. Other evidence is quoted, which suggests that vegetables should be steamed rather than boiled in water (cp. Expts. 13 and 14, Table IV) and, if boiled, should be boiled in water only, the addition of either acid (citric acid) or alkali (soda) to the water increasing the inevitable loss of antiscorbutic vitamines.

5. Experiments are also described in which autoclaved milk was omitted from the diet and in which larger rations of cabbage were given (15 to 30 g.), heated to temperatures from 100° to 130° for periods of one to two hours. The destruction of antiscurvy properties, though extensive, was found to be much less than was stated by Holst and Frölich and also less complete than would have been expected from the results of experiments in which a smaller ration and lower temperatures were employed. It is suggested that this apparent discrepancy may be in part explained by the fact that in this series of experiments both the antiscorbutic factor and the fat-soluble accessory growth factor are drawn from the same food stuff.

6. The results of experiments described in (5) throw some light on the heat stability of the fat-soluble growth factor present in green cabbage leaves. The effect of heating for 1–2 hours at temperatures from 100°–120° was slight though perceptible. After heating at 130° for two hours, however, a serious amount of destruction had taken place.

7. Further experiments which are in progress with expressed juices of fruits and vegetables given raw and after exposure to different temperatures are expected to throw more light upon the problems dealt with in (5) and (6).

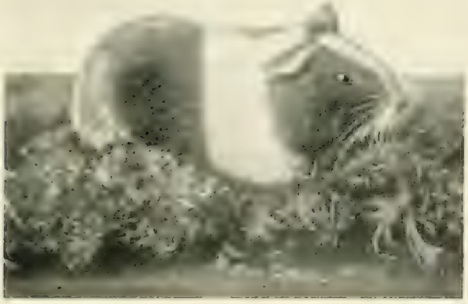

Fig. 1. No. 377. Normal after 3 months on diet of oats, bran, water, and 15 g. cabbage autoclaved 1 hr at 120°

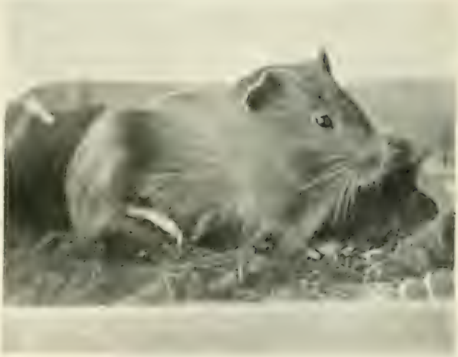

Fig. 4. No. 851. Acute scurvy showing "scurvy position" (foot) (on diet of boiled germinating lentils, unpublished experiment).

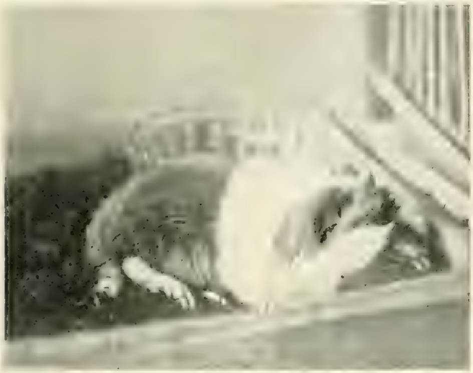

Fig. 2. No. 846. Acute scurvy, showing "faceache position" and also position of back limbs (on diet of boiled germinating lentils, unpublished experiment).

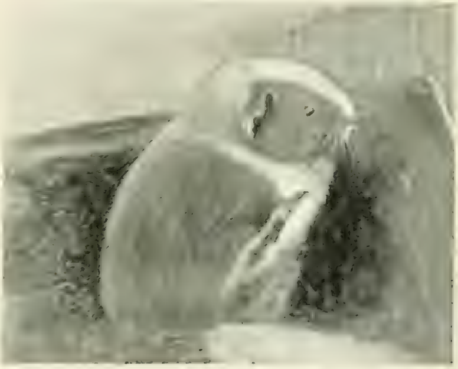

Fig. 5. Acute scurvy in animal on purely scorbutic diet of oats, bran and autoclaved milk, showing nearly vertical position of foot.

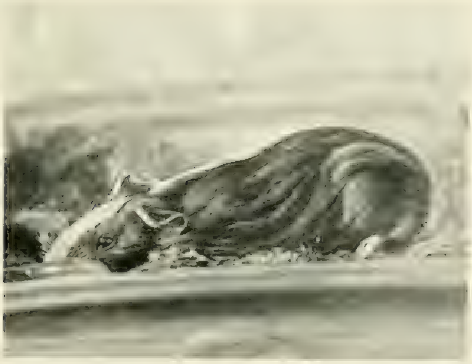

Fig. 3. No. 394. Acute scurvy showing "faceache position" after 20 days, on diet of 5 g. citric acid cabbage steamed 1 hr at 90°, oats, bran and autoclaved milk.

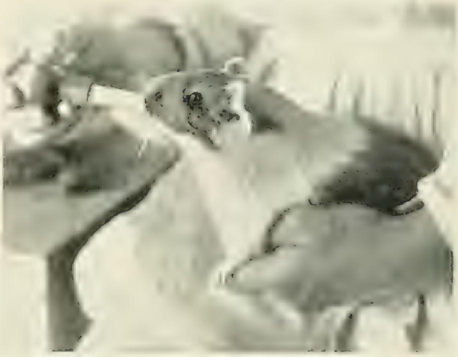

Fig. 6. No. 510. Showing method of feeding autoclaved milk to the experimental animals.

I am indebted to Dr H. Chick of this Institute for much help in carrying out these experiments, and for valuable suggestion and criticism in their interpretation. I am also indebted to Miss O. Lodge, formerly an assistant in this Department, for her care of the animals used in Expts. 4, 5 and 6; to Miss D. Gardiner for much assistance in the care of many of the experimental animals. I have also to thank my brother, Mr T. F. Delf, for his skill and patience in obtaining the photographs of Plate I.

APPENDIX

By FRANCES MARY TOZER.

The illustrations (Plate II) are diagrammatic drawings made from histological preparations of the rib-junctions of animals used by Dr Marion Delf in her series of experiments. They are intended to demonstrate the more important histological changes which are observed in animals suffering from scurvy in varying degrees.

The diagnosis made from the histological preparations is, in all cases, independent of Dr Delf's diagnosis of the condition seen during the life of the animal or at the post-mortem examination. The particulars of the experiments were not supplied until the microscope slide was made and reported on, thus eliminating any bias on the part of the observer.

The results as a rule tally closely.

The slight changes observed in the rib-junctions in cases diagnosed as "Nearly Normal" or "Incipient Scurvy" were found to correspond with general good health: in many cases growth was satisfactory, no marked symptoms of scurvy were detected during life and, with very few exceptions, departure from the normal appearance at the post-mortem examination, if any, was insignificant.

The condition found in cases described as "Definite Scurvy" was accompanied by definite symptoms of soreness and a sub-normal condition during life combined with scorbutic lesions on post-mortem examination. The more marked changes in "Acute" cases correspond with severe illness during life and usually death from the disease [see Holst, 1907; Holst and Frölich, 1912; Chick, Hume and Skelton, 1918, p. 137]. These latter authors describe the changes at the rib-junction in the following words:

"Typical changes take place in the costo-chondral junctions. They become swollen, and exhibit a transverse yellow bar.... These appearances correspond with the complete disorganisation of the bone-cartilage junction, and with disappearance of the cartilaginous trabeculae and of the rows of cartilaginous growing cells. In many cases the bone is found to be completely fractured and there is great proliferation of connective tissue across the junction."

This description applies to the "Acute" condition. "Chronic" cases are those which, though suffering from scurvy in varying degrees, are sufficiently protected to prevent death within a period of 90 days: they show, histologically, the condition seen in diagrams 5 and 6. In these cases it seems probable that the ossified band across the junction is an attempt to strengthen the junction in an abnormal manner, the normal process having broken down to an extent depending on the severity of the disease. It is therefore apparent that "Chronic" cases vary in severity according to the degree of protection given.

It is not intended to deal here with the histological changes in the bone cartilage junctions possibly caused by deficiency of the growth, or any other accessory food, factor. In case of the animals to which the following diagrams apply these factors were provided in the diet by oats and bran and autoclaved milk respectively, and it is believed that the amounts consumed were adequate. In other experiments however, where a liberal supply of an antiscorbutic was given and where the fat-soluble A growth factor was known to be deficient, the resulting histological changes in the rib-junctions of the animals examined were found to resemble closely those of "Definite" or of "Definite Chronic" scurvy. In these test cases the antiscorbutic chosen was one believed to be deficient in the fat-soluble A growth factor (for example orange juice). This fact is mentioned because in the case of an animal not receiving an adequate supply of fat-soluble A the resulting changes in the rib-junctions are not dissimilar from, and are likely to be confused with, those caused by scurvy alone.

Note added 13. 1. 19. Since this paper was written further evidence has been obtained, confirming the statement that histological conditions indistinguishable from those termed "incipient" and "definite" scurvy may be caused by an insufficiency in the diet of the fat-soluble growth factor. In cases where the diet consisted of oats, bran and water with a small cabbage ration, it is possible that the histological findings should be thus interpreted (e.g. Table II, exp. 3). Where, however, the cabbage ration, raw or heated, was large (15–30 g, Table V, expts 19 and 21) or where milk was added to the diet (e.g. Table II, expts 5 and 6, Table IV, exp. 8), we have attributed these changes to slight deficiency in the antiscorbutic factor.

Some doubt has, however, arisen as to whether the standard ration of 60 c.c. autoclaved milk may be considered an adequate source of fat-soluble growth factor for guinea-pigs in *all* cases. Further experiments, now in progress, are designed to determine with greater exactness the average amount of fat-soluble growth factor as autoclaved milk necessary for normal growth of guinea-pigs, when all other accessory factors are present in excess.

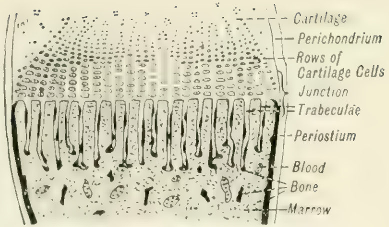

Fig. 1. Normal Rib Junction.

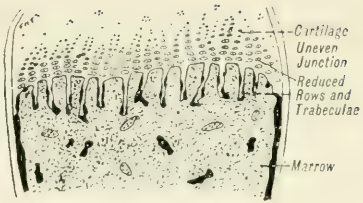

Fig. 2. Nearly Normal Rib Junction, "Incipient Scurvy."

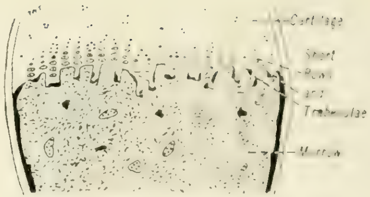

Fig. 3. "Definite Scurvy."

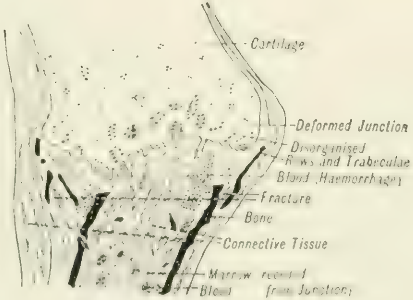

Fig. 4. "Acute Scurvy."

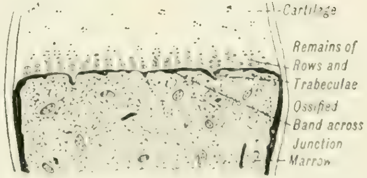

Fig. 5. "Chronic Scurvy" (definite).

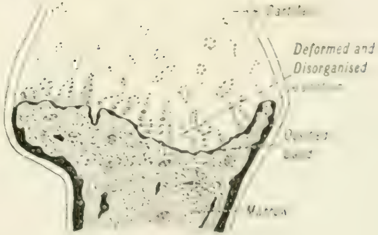

Fig. 6. "Chronic Scurvy" (acute).

DESCRIPTION OF DIAGRAMS, PLATE II.

1. *Diagram of Normal Rib-Junction* as represented by guinea-pig 823 and others.
2. *Diagram of Rib-Junction to illustrate "Incipient Scurvy,"* as represented by guinea-pigs 420–424, 379, 380, 826. This rib-junction is very nearly normal, but shows what may be regarded as the first recognisable signs of the onset of the disease; these are:
    - (*a*) An appearance of general abnormality (when compared with the normal).
    - (*b*) Unevenness of the junction and slight disorganisation of the rows of cartilage cells.
    - (*c*) Shortening of the length of the rows of cartilage cells.
    - (*d*) Shortening, and decrease in number of the trabeculae.
    - (*e*) Increased amount of blood in the marrow cavity.
3. *Diagram of Rib-Junction to illustrate "Definite Scurvy"* as represented by guinea-pigs 443, 848, 385, 391. Histological symptoms vary considerably in these cases (according to the severity of the disease), and may approach in appearance those characteristic of the "*Acute*" condition. The rows of cartilage cells may be almost normal, but are more often noticeably shortened and usually somewhat disorganised or arranged in circular groups. The trabeculae are usually about half the normal length and have a truncated appearance. The junction as a whole may present an uneven, slightly disorganised appearance and there is sometimes a certain amount of macroscopic deformity.
4. *Diagram of Rib-Junction to illustrate "Acute Scurvy"* as represented by guinea-pigs 381–384, 806–808, 847. In these cases the junction and rows of cartilage cells are often completely disorganised, causing a marked macroscopic deformity of the bone.

    The bone of the shaft is frequently fractured.

    The marrow is no longer in contact with the edge of the junction and the space is filled with a mass of connective tissue; this is very characteristic in cases of fracture. A condition in which the trabeculae have almost disappeared and the rows are much shortened is also found in "Acute Scurvy," in those ribs in which no fracture of the shaft has occurred; usually however there is little or no ossification across the junction.

    There is frequently haemorrhage into the marrow cavity.

Diagrams 5 and 6 illustrate types of "Chronic" scurvy.

5. *May be described as "Chronic Definite Scurvy"* as represented by guinea-pig 841.

    The rows are much shortened, but not disorganised and an ossified band extends across the junction.
6. *May be described as "Acute Chronic Scurvy"* as represented by guinea-pig 387.

    The junction is deformed, and the rows are very disorganised; the trabeculae have disappeared and an ossified band extends across the junction. There is no connective tissue to be seen and no existing, or recent, fracture.

    Particulars of the animals quoted by their numbers in this appendix are given in Tables VI–VIII.

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XXXIV. THE ANTI-SCORBUTIC VALUE OF CABBAGE. II. THE EFFECT OF DRYING ON THE ANTISCORBUTIC AND GROWTH PROMOTING PROPERTIES OF CABBAGE.

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(*Received November 15th, 1918.*)

In epidemics of scurvy in the past, the attempt has often been made to use dried vegetables or herbs of known antiscorbutic value. Thus in 1720 Kramer, chief surgeon to the Austrian army, in the Austro-Turkish war, was confronted with a serious epidemic of scurvy among the troops; on receiving his appeal for help, the college of physicians of Vienna sent him a great quantity, dried, of the most approved antiscorbutic herbs, but these were of no avail and thousands perished from scurvy [Lind, 1757].

Dried vegetables and herbs were repeatedly used in our own Navy for prevention of scurvy with the same result, and by the middle of the eighteenth century the fact that they could not be relied on for this purpose was well known. Lind [1757, p. 143] says "The latest proposal to the Lords of the Admiralty was a magazine of dried spinage prepared in the manner of dried hay. This was to be moistened and boiled in their food. To which it was objected by a very ingenious physician that no moisture whatever could restore the natural juices of the plant lost by evaporation, and, as he imagined, altered by a fermentation which they underwent in drying." To this a footnote is added, giving the physician's name as Dr Cockburn and stating "The Doctor's judgment is fully confirmed by experience. We find the college of physicians at Vienna sent to Hungary great quantities of the most approved antiscorbutic herbs dried in this manner; which were found to be of no benefit."

This experience was forgotten and more than a century later in the American Civil War, 1861–1865 [*Medical History*, 1888], large rations of dried vegetables allotted to the soldiers, did not prevent outbreaks of scurvy, and even at the present day, dried vegetables are still regarded by many as equivalent in value to the fresh, in spite of the tragic results of former experience. This opinion would be comparatively harmless, even if translated into action, at a time when a liberal mixed diet was available, but in case of real shortage of the antiscorbutic elements of the diet, it is of obvious importance to know how far reliance may be placed upon dried foods. This is perhaps especially true of the present winter, owing to the unusual failure of the fruit harvest, and to the limited possibilities of importing supplementary supplies.

There is, however, little experimental evidence with regard to the matter. In 1912 a paper by Holst and Frölich [1912] appeared dealing with this question among others. In an exhaustive research upon guinea-pig scurvy, its prevention and cure, these authors used potatoes, carrots, dandelion leaves and cabbage, dried in an incubator for some days at 37° or *in vacuo* at 30°. The material was stored for six months or a year at summer temperature mostly in closed vessels, but in one case in open vessels in an incubator, thus preventing any acquisition of hygroscopic water.

When these vegetables were fed to guinea-pigs in the fresh condition, daily rations of 30 g. added to a dry grain and water diet maintained the animals in health and prevented scurvy. In the case of the dried cabbage, the material was soaked in water for half-an-hour before being fed to the animals, amounts corresponding to the dry weight of 30 g. fresh cabbage being allotted to each animal. Any water unabsorbed was fed to the animals separately. The remaining elements of diet consisted of oats and bran *ad libitum*, and water.

Six animals receiving cabbage dried and stored in the laboratory for one year, died with symptoms of acute scurvy in 21–28 days. On an entirely scorbutic diet consisting of grain and water only, guinea-pigs of the age used in these experiments die of acute scurvy in 20–30 days. It is thus seen that no protection was afforded by the dried cabbage. Another eight animals receiving cabbage dried and stored for six months, died with acute scurvy in 21–37 days; these also received no significant protection from the disease.

A few additional observations were made with the cabbage dried at 37° and stored in an incubator in an open vessel. After six months of such storage a certain amount of protection was afforded by a daily ration equivalent to 30 g. of fresh cabbage to three animals which, however, died ultimately of acute scurvy in 51 to 71 days. Better results were obtained when the cabbage had been stored only six weeks. On this diet two animals died of scurvy after 66 days and two lived for 98 days, one with no apparent symptoms and the other with evidence of scorbutic lesions on histological examination of the bony tissues.

In a later experiment [Holst and Frölich, 1913] scurvy was prevented in case of two animals for 84 and 62 days respectively with a ration of dried cabbage equivalent to 30 g. fresh; but in this case the cabbage was dried at 37° and kept at that temperature in a desiccator over sulphuric acid. In this case also, as in the other experiments of Holst and Frölich, no increase in weight of the animals took place during the period of experiment.

TABLE I. *Summary of results of Holst and Frölich.*

Ration of Cabbage equivalent to 30 g. fresh cabbage; the rest of the diet consisted of oats, bran and water.

Stored	Age at beginning of experiment	No. of animals	General result
Closed bottles in laboratory	1 year	6	Death from acute scurvy (21–28 days)
do. do.	6 months	8	,, ,, (21–37 days)
Open vessel in incubator at 37°	6 ,,	3	,, ,, (51–71 days)
do. do.	6 weeks	4	One lived 98 days, with no apparent symptoms ,, ,, 98 ,, all bones scorbutic Two ,, 66 ,, all bones scorbutic, also with haemorrhages
Desiccator over sulphuric acid at 37°	4 months	2	One lived 84 days—no symptoms seen in life or at post-mortem One died after 62 days—no symptoms seen in life or at post-mortem

These experiments indicate that the time of storage has a marked effect upon the antiscorbutic value of dried cabbage. This value was greater when the dried material was kept in a very dry atmosphere and prevented from taking up moisture from the air. But even in this case the value is very small in comparison with the fresh material. These workers succeeded in preventing onset of scurvy in guinea-pigs with as small an amount of fresh cabbage as 1 to 2·5 g. daily, and the most successful of their experiments with dried cabbage do not suggest that more than a minimal amount of the original antiscorbutic substance had escaped destruction.

The results obtained by Holst and Frölich with dried cabbage are summarised in Table I. Their experiments with other dried vegetables showed similar deterioration on drying and storing, this being greatest with dandelion leaves and carrots, and least with potatoes. This is in accord with a general opinion held among seamen and travellers as to the practical value of dried potatoes. Instances are not lacking, however, in which their inclusion in a diet has failed to prevent scurvy [*Medical History*, 1888].

First Series of Experiments with Dried Cabbage.

Preliminary experiments with dried cabbage were carried out by one of us (R. F. S.) in the winter of 1916–1917. In July 1916 Dr Harden kindly prepared a stock of material for us dried in a hot room at 37° C. He thought it might be worth while to try the effect of killing the plant cells with boiling water previous to the drying. Such an operation would inactivate any oxidases or other enzymes, present in the cells; these bodies if present, undestroyed, would remain active for a time during the drying process and might be partly responsible for the destruction of the antiscorbutic factor. The results of Holst and Frölich indicate such a possibility, for they achieved a certain amount of protection when the dried cabbage was maintained in an absolutely dry atmosphere, a condition which also would be inimical to any enzyme activities.

Two samples of dried cabbage were therefore prepared; one in which the fresh material was dried in a hot room at 37° forthwith, the other in the preparation of which the fresh material was plunged into boiling water for five minutes previous to drying. Both samples contained about 10 per cent. residual moisture, estimated by drying at 100°. Feeding experiments were started four to six months later, the dried material having been stored meanwhile at laboratory temperature (about 15·5°) in stoppered jars.

Both samples were fed to the animals after moistening with water overnight. The dry weight given was at first the equivalent of 30 g. fresh cabbage, and later double and treble this amount was given. The soaked cabbage was usually eaten well, but when this was not so, a rough estimate was made by eye of the amount left uneaten.

The results of these experiments are shown in tabular form in Tables II and III and illustrated by typical weight curves in Fig. 1. It will be seen by reference to the tables, that both samples of dried cabbage, when offered in amount corresponding to 30 g. fresh cabbage, failed to protect the animals from scurvy. Those on the unboiled sample died with all the symptoms of acute scurvy in 24 to 31 days, showing that they had received no protection. Those on the boiled sample died in a similar manner in 23 to 66 days, some having received distinct protection. These results are in close agreement with those of Holst and Frölich who found no protection in their dried cabbage after six months' storage at laboratory temperature, when no particular precautions were taken as regards access of moisture.

Other animals were offered double and treble the above ration, in the

TABLE II. *Experiments with dried cabbage, unboiled.*

Number of animal	Rations					Weight, g.			Duration of experiment, days	Symptoms in life	General conclusions
	Dried cabbage offered, g.	Equivalent in fresh cabbage, g.	Dried cabbage consumed, g. average	Equivalent in fresh cabbage, g. average	Oats and bran consumed, g. average	Initial	Final	± %			
25	3·2	30	3·2	30	—	340	210	−38	31	"Faceache position" on 17th day	Death from severe scurvy on 31st day
26	3·2	30	irregular	—	—	390	230	−41	28	Tender joints on 18th day	Death from severe scurvy on 28th day
27	3·2	30	3·2	30	—	445	270	−39	28	"Faceache position" on 18th day	Death from severe scurvy on 28th day
28	3·2	30	3·2	30	—	265	180	−32	24	Quiet and flabby	In pain, chloroformed on 24th day; post-mortem, severe scurvy
36	6·4	60	6·4	60*	30	350	325	− 7	33	"Faceache position" on 22nd day	In pain, chloroformed on 33rd day; post-mortem, severe scurvy
101	6·4	60	5·0	47*	32	310	215	−31	26	Sore knees on 18th day	Death from severe scurvy
102	6·4	60	5·0	47*	32	295	220	−25	30	Scurvy position and swollen knees on 23rd day	Diarrhoea, chloroformed on 30th day; post-mortem, severe scurvy
98	9·6	90	7·6	71*	32	300	235	−22	25	Tender joints on 12th day	Moribund, chloroformed on 25th day; post-mortem, slight scurvy
99	9·6	90	8·0	75*	28	270	270	0	23	Tender joints on 14th day	Acute attack, fits; chloroformed; post-mortem, slight scurvy
100	9·6	90	9·6	90	31	345	395	+14	61	"Scurvy position" on 28th day	Abscess in thigh, chloroformed; post-mortem, very slight scurvy

* indicates approximate amounts.

TABLE III. *Experiments with dried cabbage, boiled*

Number of animal	Rations					Weight, g.			Duration of experiment, days	Symptoms in life	General conclusions
	Dried cabbage offered g.	Equivalent in fresh cabbage, g.	Dried cabbage consumed, g. average	Equivalent in fresh cabbage, g. average	Oats and bran consumed, g. average	Initial	Final	± %			
22	2·6	30	2·6	30	23	390	300	−23	58	"Faceache position" on 22nd day	Leg broken, chloroformed on 58th day; post-mortem, severe scurvy
23	2·6	30	1·3	15*	—	265	180	−32	23	None	Moribund, chloroformed on 23rd day; post-mortem, severe scurvy
33	2·6	30	1·9	21*	26	310	270	−13	34	Tender joints from 21st day	In pain, chloroformed on 34th day; post-mortem, severe scurvy
35	2·6	30	2·6	30	21	320	205	−36	66	"Faceache position" on 20th day, tender joints later	Died on 66th day; chronic scurvy, crippled and undergrown
34	5·2 7·8	60 90	5·2 7·8	60 106 days 90 28 ,,	42	390	340	−13	134	Knees tender on 34th day	On 127th day gave fresh cabbage, became ill, chloroformed on 134th day; post-mortem, chronic scurvy
96	5·2	60	4·8	55*	29	310	390	+26	66†	Sore joints on 23rd day	Good condition, only slight symptoms in life; chloroformed on 66th day; post-mortem, no scurvy
97	5·2	60	3·8	44*	26	290	345	+19	66†	Sore joints on 14th day	Fair condition; only slight symptoms in life; chloroformed on 66th day; post-mortem, very slight scurvy
93	7·8	90	7·0	80*	44	295	170	−44	34	Sore joints on 26th day	Moribund, chloroformed on 34th day; post-mortem, severe scurvy
94	7·8	90	7·3	84*	30	300	478	+59	66†	"Scurvy position" on 27th day	Splendid condition after recovery from slight attack of scurvy about 27th day; returned to stock
95	7·8	90	7·0	80*	36	325	300	− 8	66†	Tender on 16th day	Good condition but failed to grow; post-mortem, no scurvy

* indicates approximate amounts.
† indicates experiment stopped owing to lack of material for rations.

hope of achieving complete protection. In the case of the double ration of unboiled dried cabbage (*i.e.* equivalent to 60 g. fresh cabbage) death from scurvy resulted in 26 to 33 days, thus again implying little or no protection, but on the double ration of dried boiled cabbage, the result was distinctly better. One animal lived for about 70 days in a condition of chronic scurvy and two others lived for 66 days exhibiting distinct soreness of the joints, the experiment being then terminated by the lack of further supplies of the dried cabbage. A review of the symptoms in life and of the post-mortem appearances shows that these three animals were suffering from scurvy in a more or less acute form. The protection was therefore partial, but was certainly greater than was received by the animals on a similar ration of the unboiled dried cabbage.

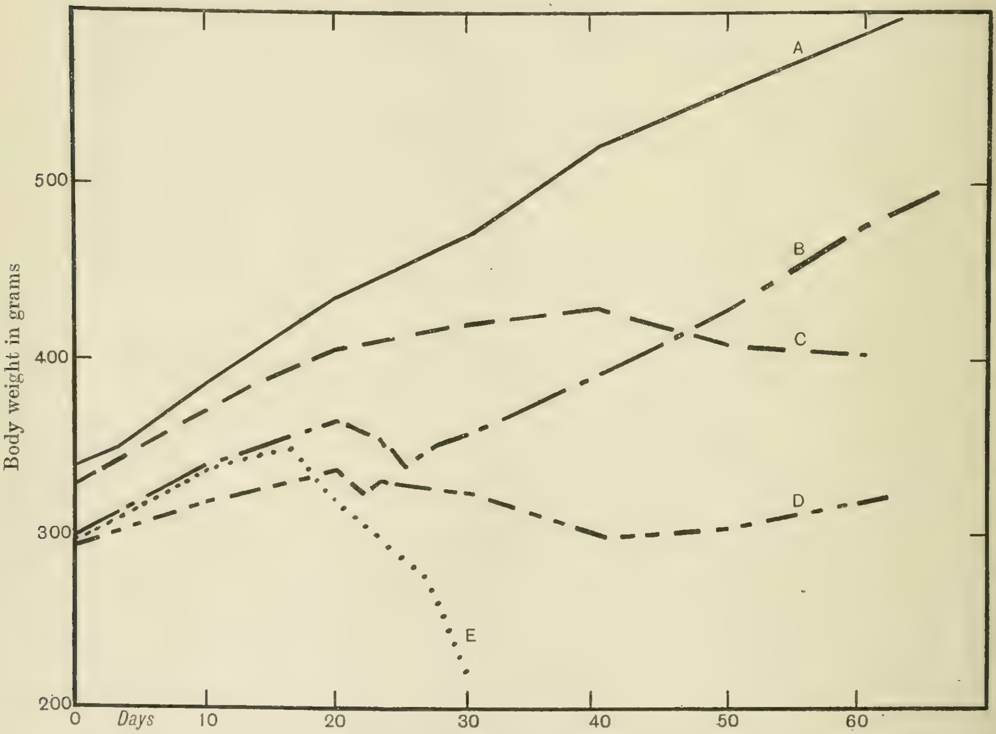

Fig. 1. Comparison of growth on fresh and dried cabbage. Selected curves illustrating preliminary experiments with dried cabbage. The rest of the diet consisted of oats, bran and water ad lib.

——— Curve A represents standard growth on a 30 g. fresh cabbage ration.

— • — • ,, B represents growth of No. 94, an animal of exceptional vigour, on 7·3 g. dried boiled cabbage (=84 g. fresh).

— — — ,, C represents growth of No. 100 on 9·6 g. dried unboiled cabbage (=90 g. fresh).

— • • — • • ,, D represents growth of No. 95 on 7 g. dried boiled cabbage (=80 g. fresh).

• • • • • • ,, E represents growth of No. 102 on 5·0 g. dried unboiled cabbage (=47 g. fresh).

On the treble ration (*i.e.* equivalent to 90 g. fresh cabbage) the full amount was not always eaten. Symptoms of scurvy appeared in every case during life, but here again, those on the boiled sample appeared to thrive better than those on the unboiled, and in two cases were in good health upon the 66th day of the diet.

These experiments confirm the general conclusion of Holst and Frölich, that dried cabbage when stored for six months or longer under ordinary conditions of temperature and moisture, offers very little protection if given as the sole antiscorbutic in a guinea-pig's diet. They also indicate that killing the cells by heat before drying is beneficial in lessening the amount of destruction taking place during drying.

This result, taken in conjunction with that of Holst and Frölich, in which dried cabbage was shown to preserve its remnant of antiscorbutic virtue the better if kept in an absolutely dry atmosphere, lends support to the view that some, at least, of the destruction may be due to the activity of an enzyme or other body originally present in the living tissue.

From the results of Holst and Frölich combined with those of control experiments with fresh cabbage made by one of us [Delf, 1918] in connection with another set of experiments we may conclude that a daily ration of about 1 g. fresh cabbage is the minimum amount required to protect a guinea-pig from scurvy upon an otherwise "scurvy diet." In the most favourable cases described above, therefore, we may say that a ration of dried boiled cabbage, equivalent to 60 g. raw cabbage, after four to six months' storage, has a protective value which is less than that of 1 g. fresh raw cabbage, *thus representing a loss of antiscorbutic value of at least 98 per cent.*

EXPERIMENTS WITH COMMERCIALLY DRIED CABBAGE.

In 1917 as part of Food Economy propaganda repeated attempts were made in the daily press to encourage the drying of fresh vegetables. Various articles appeared which implied that the dried material was equivalent in value to the fresh. It therefore seemed desirable to determine more exactly the antiscorbutic and growth promoting properties of fresh and dried cabbage respectively. These experiments were undertaken by one of us [E. M. D.] in 1918, before giving publicity to the results already obtained.

By the courtesy of Mr J. F. Martin, weekly samples of dried cabbage were sent regularly from the Dunnington Farm Produce Factory. These were at first savoys (Feb. 5 April 9, 1918) and later spring cabbages (April

15–June 4, 1918). They were dried without previous treatment, at 120–150° F. (*ca.* 60° C.) for 3–4 hours in an air current driven by electric fan, a process which is essentially that adopted for commercial purposes. The samples were usually received the day following the drying and were stored in stoppered or covered jars in the laboratory at a temperature of 60–65° F. They were dated and used in succession, so that a particular group of animals always received samples of the same age. The ages selected were (*a*) 2–3 weeks, (*b*) 5–6 weeks, and (*c*) 12–14 weeks, in the different series of experiments. The water content varied slightly in the different samples, but averaged about 10 per cent. as before.

Control experiments with raw cabbage leaves.

In a previous communication in this Journal [Delf, 1918] control experiments are described in which young guinea-pigs were kept for a period of 90 days on a diet of oats, bran and varying rations of fresh raw cabbage. The greatest amount of growth was made on a 30 g. daily ration of cabbage (about 90 per cent. increase in body-weight); rather less growth was made on a 15 g. ration (about 70 per cent. increase in body-weight) and still less on a 5 g. ration of cabbage (about 26 per cent. increase in body-weight). Since all these animals were protected from scurvy, it appeared that the limitation of the cabbage ration was reducing too greatly the fat-soluble accessory growth factor known to be present in the cabbage leaves [McCollum and Davies, 1915], the water-soluble growth factor having been abundantly supplied in the cereal part of the diet [McCollum, Simmonds and Pitz, 1917]. Some animals were therefore given a daily ration of milk previously autoclaved for an hour at 120° in addition to a small cabbage ration; 5 g., 2·5 g., 1·5 g. or 0·5 g. cabbage. There is independent evidence that the addition of such a ration of milk improves greatly the nutritive value of the diet without affecting the onset of scurvy [Chick and Hume, 1917; Chick, Hume and Skelton, 1918]. With a diet of 5 g. raw cabbage, oats, bran and 60 cc. autoclaved milk daily, the average growth of the experimental animals was nearly as great as on a diet of oats, bran and 15 g. raw cabbage. With a smaller cabbage ration (2·5, 1·5 g.) the growth was less, but there was no development of scurvy. When the cabbage ration was reduced to 0·5 g. daily, definite symptoms of scurvy were developed in three out of four cases, one animal succumbing to the disease after 50 days. The minimum daily ration of fresh raw cabbage necessary to protect a young guinea-pig from scurvy upon this diet was therefore taken as about 1 g. daily.

Table IV. *Experiments with Dunnington Farm Dried Cabbage.*

Exp.	No. of animal	Sex	Ration	Age of sample, weeks	Weight of animal, g.		Gain % on initial weight	Duration of exp., days	Symptoms	Post-mortem	Histology of rib-junctions	Average amount of food consumed daily			Condition	General conclusion
					Initial	Final						Oats and bran, g.	Dried cabbage, g.	Aut. milk, cc.		
1	813		3·4 g. dried (= 30 g. fresh)	2–3	312	404	29	90	Lameness from 42nd day	Bones somewhat fragile; 9 rib-junctions ridged	Acute scurvy	48	3·4	—	Thin, but active	Chronic scurvy (killed* after 90 dys)
	814	♂			305	400	31	90	Scurvy position from 27th day	Bones fragile; haemorrhages in muscles and subcutaneous tissue. 9 rib-junctions ridged	do.	41	3·4	—	do.	do. do. do.
	815	♂			352	350	0	52	Soreness from 26th day, persistent	Bones very fragile; severe muscular and subcutaneous haemorrhages; 5 rib-junctions nodular and swollen	do.	45	3·4 (26 dys) 3·1 (26 dys)	— —	Wasted	Acute scurvy (died)
	816				350	250	−29	48	Scurvy position from 31st day	Bones very fragile; severe haemorrhages; all rib-junctions swollen, and nodular	do.	28	3·3 (31 dys) 1·7 (17 dys)		do.	do.
2	824	♂	3·4 g. dried (= 30 g. fresh)	5–6	350	280	−20	59	Soreness from 31st day	Bones very fragile; haemorrhages severe	do.	36	3·4 (30 dys) 2·7 (29 dys)		do.	do.
	825	♀			360	230	−36	52	,, ,, 23rd ,,	do. do. do.	do.	23	3·4 (22 dys) 2·6 (30 dys)	— —	do.	do.
	825 A				345	488	+41	82	,, ,, 20th ,,	Bones firm, 4 rib-junctions ridged; slight muscular haemorrhage	Nearly normal	23	3·4 (82 dys)	—	Good	Slight scurvy (killed* after 82 dys)
	827				355	210	−41	48	,, ,, 19th ,,	Bones very fragile; severe haemorrhages	Acute scurvy	32	3·4 (48 dys)	—	Emaciated	Acute scurvy (died)
	828				325	234	−28	55	,, ,, 19th ,, (scurvy position)	do. do. do.	do.	24	3·4 (55 dys)	—	do.	do.
3	842		1·7 g. dried (= 15 g. fresh) + autoclaved milk	2–3	340	475	+40	88	Soreness, and scurvy position from 25th day	Bones firm; signs of previous fracture of knees; 3 rib-junctions ridged	Slight scurvy	33	1·7	59	Good	Chronic scurvy (killed* after 88 dys)
	843	♂			330	342	4	80	Soreness, and scurvy position from 24th day	Bones firm; previous fracture and haemorrhage of knees	do.	18	1·5–1·6	56	do.	,, ,, 80 ,,
	844				325	530	+63	83	Soreness, knees swollen, from 17th day	do. do. do. do.	do.	30	1·6–1·7	60	do.	,, ,, 83 ,,
	845				350	480	+37	83	Soreness (ribs and knees) from 26th day	Bones firm; previous fracture of knees	Scurvy	31	1·7	60	do.	,, ,, 83 ,,
4	877		1·7 g. dried (= 15 g. fresh) + autoclaved milk	12–14	330	400	+21	93	Soreness, scurvy position from 25th day, later very lame	Tibiae soft, femur hard; knees severely haemorrhaged; severe subcutaneous haemorrhages; all rib-junctions ridged	do.	14	1·6 (93 dys)	56	do.	,, ,, 93 ,,
	878	♂			335	225	−33	56	Soreness, swollen joints, from 17th day	Bones very fragile; severe subcutaneous and muscular haemorrhages; all rib-junctions ridged	Acute scurvy	8	1·6 (28 dys) 1·1 (28 dys)	56	Emaciated	Acute scurvy (died after 56 dys)
	879	♀			330	225	−32	52	,, ,, ,, ,, 21st ,,	Bones very fragile; knees fractured; extensive haemorrhages; all rib-junctions ridged	do.	8	1·6 (28 dys) 0·8 (24 dys)	57	do.	Acute scurvy (died)
	880	♂			350	455	+30	90	,, ,, ,, ,, 23rd ,,	Bones very fragile; haemorrhages severe (both muscular and subcutaneous). All rib-junctions markedly ridged	Scurvy	13	1·6 (90 dys)	58	Good	Scurvy

* By chloroform.

This series of experiments with graded rations of raw cabbage with and without milk [Delf, 1918] forms the basis of the present attempt to determine more precisely the effect of drying on (*a*) the antiscorbutic and (*b*) the growth promoting factors present in the cabbage leaves.

Experiments with Dunnington Farm Greens.

Table IV gives a *résumé* of the experiments made with the Dunnington Farm dried cabbage samples, oats and bran forming the remainder of the diet in all cases.

1. *Dried cabbage 2–3 weeks old* (*ration equivalent to* 30 *g. fresh cabbage with oats and bran*). Exp. 1, Table IV.

Four animals fed with the dried cabbage 2–3 weeks old were given a daily ration of 3·4 g., equivalent to 30 g. fresh cabbage. This was readily eaten at first, and, in two cases, throughout the experiment. After 26–42 days all the animals developed scorbutic symptoms and two died (Nos. 815, 816) of acute scurvy in 48 and 52 days respectively. In both these cases, the appetite for the dried cabbage diminished towards the end of the experiment, but in each case not until after the appearance of the first scurvy symptoms (cp. Table IV). This lack of appetite no doubt hastened the last stages of the disease, and was probably caused by the loose and brittle teeth afterwards found at the post-mortem examination. However the disease was definitely recognised in the early stages, before any falling off of appetite had occurred. The two remaining animals survived in fair condition for 90 days when the experiment was terminated. Both post-mortem and histological examination revealed a condition of chronic scurvy, and the animals would probably have survived much longer in a permanently scorbutic condition.

There is here less protection from scurvy than would be expected from a ration of 1 g. fresh cabbage daily, that is to say, the loss of antiscorbutic value due to drying is more than $\frac{29}{30}$ths of that originally present in the fresh state, *i.e.* about 97 per cent.

If we consider the body-weight in the four animals at the beginning and end of the experiment, a net increase of only about 6 per cent. is shown on the total initial weight. It is, however, difficult to generalise from four experiments in which the result as regards growth was so very divergent. In fig. 2 are set out the average growth curves of the animals in Expts. 1–4. Curves B, C, represent the mean growth of the two pairs of animals in Exp. 1. Curve C represents the loss of weight shewn by Nos. 815, 816, which died of scurvy: in the more favourable cases (Nos. 813 and 814) which survived for the whole three months of the experiment, the total gain in body-weight was about 30 per cent.

This is not much greater than was found with animals receiving a 5 g. ration of fresh raw cabbage, supplemented only by oats, bran and water; in the latter instance the average gain in weight after three months was 26 per cent. of the initial [Delf, 1918]. On this basis we may assume the growth promoting power of 30 g. fresh cabbage, 2–3 weeks after drying, to be reduced to the equivalent of about 5 g. fresh cabbage, *i.e.* about $\frac{5}{6}$ or 86 per cent. of the original, to have been lost. All the animals in Exp. 1, however, developed scurvy and the limitation of growth was probably due to the mutual effect of limited antiscurvy factor, as well as limited fat-soluble growth factor. In a later experiment (Exp. 3) autoclaved milk was added to the diet and the ration of dried cabbage was halved. Scurvy developed in every animal, but the average growth was much better than in Exp. 1. We may therefore conclude that the inferior growth in Exp. 1 must be mainly referred to deficiency in fat-soluble growth factor and that an estimate of 86 per cent. loss through drying and keeping for 2–3 weeks may be approximately correct.

2. *Dried cabbage 5–6 weeks old* (*ration equivalent to* 30 *g. fresh cabbage, oats, bran and water*). Exp. 2, Table IV.

Five animals were fed on a ration of 3·4 g., equivalent to 30 g. fresh cabbage, but the dried material had been stored 5–6 weeks. It will be seen from Table IV that symptoms of scurvy developed earlier than in the previous cases, four out of the five dying of acute scurvy. The fifth animal was chloroformed after 82 days when the experiment terminated as the supply of dried material had ended. This animal had exceptional resistance and was only slightly scorbutic when killed. On the whole, however, this set of animals exhibited much less protection than those fed on the samples 2–3 weeks old.

3. *Dried cabbage 2–3 weeks old* (*ration equivalent to* 15 *g. fresh cabbage, with oats, bran and autoclaved milk*). Exp. 3, Table IV.

It is clear from the curves B and C (Fig. 2) that on a diet of oats, bran, dried cabbage and water, there is very little growth increment even when the cabbage is recently prepared and the ration equivalent to 30 g. fresh cabbage, and this shows that during drying the growth promoting constituents

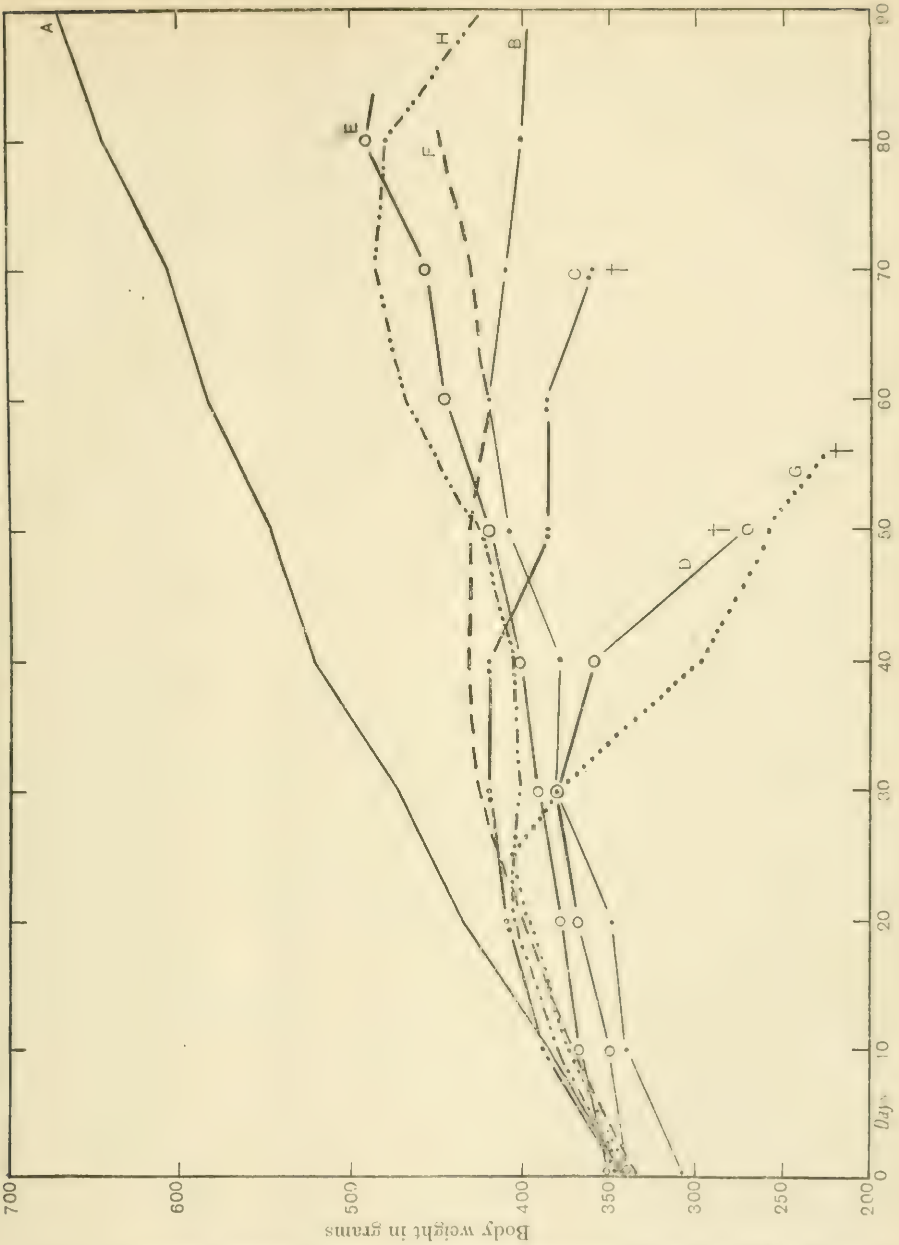

Fig. 2. Comparison of growth on fresh and dried cabbage rations (Dunnington Farm Greens).

——— Curve A represents standard growth on a 30 g. fresh cabbage ration.

Curves B and C represent average growth on diet of 3·4 g. dried cabbage (equivalent to 30 g. fresh) 2-3 weeks old and water.

—•— Curve B represents the mean growth of Nos. 813, 814 in 90 days.

—••— ,, C represents the mean growth of Nos. 815, 816, which both died of scurvy.

Curves D and E represent growth on a diet of 3·4 g. dried (equivalent to 30 g. fresh) cabbage 5–6 weeks old and water.

—○— Curve D represents the mean growth of Nos. 824, 825, 827 and 828.

—○— ,, E represents the mean growth of No. 825 A, an animal of exceptional resistance.

— — — ,, F represents average growth on 1·7 g. dried (equivalent to 15 g. fresh) cabbage 2–3 weeks old with autoclaved milk. This is the mean of Nos. 842, 843, 844, 845.

Curves G and H represent the average growth on 1·7 g. dried (equivalent to 15 g. fresh) cabbage 3 months old with autoclaved milk.

•••••• Curve G represents the mean growth of Nos. 878, 879, both dying of scurvy.

of the fresh leaves have suffered destruction as well as the antiscorbutic factor. In the following experiments, therefore, an attempt is made to distinguish these two effects by increasing the supply of fat-soluble growth factor with a ration of 60 cc. autoclaved milk (120° for one hour).

The results of these experiments proved of extreme interest. The animals made an amount of growth at first nearly equal to that achieved by a ration of 5 g. fresh cabbage with the addition of autoclaved milk (F, Fig. 2). Definite symptoms of scurvy appeared after 17–24 days causing very obvious discomfort. After about three weeks in this condition the animals gradually improved; they became less sore, regained a considerable amount of their former activity and appeared to make an almost complete recovery. At the close of the experiment it was found on post-mortem examination that all had previously suffered from fractured knees and from haemorrhages in the muscles. There is no doubt that the addition of the milk had so much improved the general nutrition of the animals as to ameliorate the severity of the scurvy symptoms.

If we attempt to estimate the amount of antiscorbutic factor left in this ration of dried cabbage, we must place it at less than the equivalent of 1 g. fresh raw cabbage, i.e. *the loss by drying and storing is at least* 93 *per cent.*

This experiment is of interest, in that it shows clearly the effect of a more nourishing diet in protecting from the most severe symptoms of scurvy, in spite of the reduction of the dried cabbage ration to one half the amount which, in the absence of milk, was found to produce death from acute scurvy. This fact is analogous to cases of human scurvy where inferior conditions of diet and comfort have been found to be directly connected with the severity of the attack. Thus when outbreaks of scurvy occurred on board ship, the crew, subjected to greater hardship and to poorer diet than the officers, were commonly attacked with greater severity, even when the amount of fresh food was apparently the same in both cases. It must be admitted, however, that in the greater variety of food enjoyed by the officers, there may also have been found additional traces of the antiscorbutic factor.

4. *Dried cabbage* 12–14 *weeks old* (*ration equivalent to* 15 *g. fresh cabbage, with oats, bran and autoclaved milk*). Exp. 4, Table IV.

Finally, a set of animals was fed on a ration of 1·7 g. dried cabbage 12–14 weeks old (equivalent to 15 g. fresh cabbage) with the addition of autoclaved milk. These were at first particularly strong and healthy animals, but all developed marked symptoms of scurvy in 17–25 days from the beginning of the experiment. One died of acute scurvy in 52 days, and another in 56 days, the remaining two surviving in a condition of chronic scurvy till the experiment was terminated, at the end of three months. All formed a striking contrast to the animals fed on a similar ration 2–3 weeks old. There is clearly very little protection from scurvy to be gained in this cabbage after three months' storage, however favourable may be the conditions of the animals. The residual antiscorbutic value is indeed less than that provided by a ration of 0·5 g. fresh raw cabbage, representing thus a loss of antiscurvy value equal to 14·5 out of 15, *i.e.* 96–97 *per cent.* The preliminary growth is checked with the appearance of symptoms of scurvy and it seems that lack of the antiscorbutic vitamine is here acting as an inhibiting factor in presence of an ample supply of both essential accessory growth factors in the diet.

From a comparison of the results of Exp. 1 and 2 with those of Exp. 3 and 4, it is clear that the effect of drying fresh cabbage leaves is twofold in destroying the greater part both of the antiscorbutic and of the fat-soluble growth factor. The effect of storage at laboratory temperatures is to cause a further progressive destruction of what antiscorbutic value is left after drying. After three months, the destruction seems to be nearly complete and the product would probably be quite valueless after six months or longer. Probably cold storage would lessen this deterioration, but in view of the great initial deterioration by drying, the value of the product is not worth the considerable expense involved in practice both in drying and in cold storage.

It is important to note that the destruction of the growth factor as also of the antiscorbutic factor, though relatively very great, is not complete. Recent experiments [Hess and Unger, 1918] have been brought forward which prove that hay possesses growth-promoting capacity, and Cohen and Mendel [1918] state that they obtained a "cure" on addition of dried cabbage to the diet of an animal which had developed scurvy on an insufficient ration of fresh milk. With regard to the former statement, we have already noticed this growth-promoting power of hay in the course of some unpublished experiments, but this capacity is doubtless but slight compared with that of the fresh grass from which it is prepared. As it has not proved practicable to obtain control samples of fresh and dried hay of the same plant composition and of known age, no critical experiments on this line have been attempted. With regard to the "cure" reported by Cohen and Mendel on addition of dried greens, the total amount of antiscorbutic in the diet, derived both from the fresh milk ration and from the dried cabbage (1 g. increasing to 3 g. dry, *i.e.* an amount equivalent to about 10–25 g. fresh), may well have reached the protective value claimed.

Another point of interest is the claim of McCollum and Davis [1915] that the drying of milk does not destroy the growth promoting power of the resulting powder. This result has been confirmed by other workers in this Institute, but it is not yet apparent to what *extent* the great growth promoting powers of fresh milk are being retained. In working with a food so rich in the fat-soluble growth factor, the amount of destruction can only be detected or estimated after careful determinations have been made of some minimum amount which, when given in the fresh and dried state respectively, just allows a selected margin of growth.

SUMMARY AND GENERAL CONCLUSIONS.

1. There is a loss in antiscorbutic properties of more than 93 per cent. when cabbage is dried at a low temperature and stored subsequently for 2–3 weeks at laboratory temperature.
2. After drying and storing for 5–6 weeks at laboratory temperature, a further loss of antiscorbutic properties is suffered.
3. After storage for three months, nearly all the protective value of the fresh material is lost (about 96 or 97 per cent.). The residual amount is distinctly greater if the cabbage is plunged into boiling water before drying.
4. In the drying and storing there is also destruction of the fat-soluble growth promoting accessory factor. This loss has been estimated at about 86 per cent. of the original value after only two weeks' storage.
5. Any remaining antiscorbutic value in dried cabbage is best utilised in the presence of an otherwise nutritious diet such as was afforded by the addition of autoclaved milk to the diet of oats and bran in the above experiments.
6. The preservation of vegetables by drying is an unprofitable process having regard to the labour and fuel involved, to the low nutritive and antiscorbutic value of the product, and to the subsequent deterioration on storage.

In conclusion, our best thanks are due to Mr J. F. Martin of the Dunnington Farm Produce Factory for the trouble taken in dispatching regular weekly supplies of dried cabbage over a period of several months, also to the Ministry of Food, Preserving Section, for kindly making the necessary arrangements. We also wish to acknowledge the help of Miss D. G. Gardiner who has assisted in the tedious work involved in the care and feeding of the experimental animals.

NOTE.

Since writing this paper, an article has appeared on the "Antiscorbutic Property of Desiccated and Cooked Vegetables" [Givens and Cohen, 1918].

The authors used a basal diet of heated soy bean flour, milk, yeast, paper pulp, sodium chloride and calcium lactate, incorporated into a mixture which was subsequently dried before feeding to the experimental animals (guinea-pigs of various ages). The dried vegetables were cabbage and potatoes, the former being slowly air dried at temperatures of 38–52° ("low" dried) or 75° ("high" dried) or minced and boiled 20 minutes in water before drying. The potato was given after mincing, boiling half-an-hour and drying slowly, on radiators.

The experiments were made with these vegetables given to the extent of about 1 g. daily, in addition to the soy bean diet. The "high" dried cabbage, the cooked dried cabbage and potato were found to be of no antiscorbutic value. The low dried cabbage was found to give partial protection for 59–81 days if consumed at the rate of not less than 1 g. daily. In the most favourable cases life was prolonged for 78 and 81 days respectively, and the post-mortem examination of these animals indicated only slight scurvy: no histological examination of the bones was made.

This result is in accord with our Exp. 3, Table IV, but in the histological examination the scorbutic condition of the animals was still further emphasised.

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XXXV. THE EXTENSION OF THE GAS LAWS TO LIQUIDS AND SOLIDS. I.

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1. *The necessity of re-stating the Gas Laws.*

It was shown by van 't Hoff from Pfeffer's experimental data that in the case of dilute solutions of sugar and other non-electrolytes the osmotic pressure produced by the substance in solution is practically the same as the ordinary outward pressure it would exert if present by itself as gas at the same temperature and in the same concentration per unit volume as in the solution. In other words, if osmotic pressure is taken to represent gas pressure, Boyle's, Charles's or Gay Lussac's and Avogadro's laws for gases can be extended in some sense to substances in dilute solution. Nevertheless there is still no agreement as to how osmotic pressure can represent gas pressure, or how, if it does, the pressure is not in the opposite direction.

A very clear picture of the great divergence of opinion is afforded by the recent discussion on Osmotic Pressure at a meeting of the Faraday Society in May 1917 [Faraday Society, 1917]. Professor Porter defends van 't Hoff's kinetic theory that osmotic pressure is the bombardment pressure of the solute molecules, and therefore, in solutions sufficiently dilute to prevent complicating conditions, has the same value as if the sugar were present as a gas at the same temperature and concentration, with nothing else present in the same space, or only another gas at an ordinary pressure. He points out that the kinetic theory "is the *only one* which directly gives the experimentally ascertained values for dilute solutions." He is forced, however, to make the assumption that molecules may be treated, not as "real molecules," occupying space, but as "massive points" without extension. Only on this condition does the van 't Hoff theory yield the experimental values for dilute solutions. As was pointed out by other contributors, we cannot, in view of the experimental facts as to highly compressed gases, make any such assumption. Moreover the osmotic pressure on this theory is in the wrong direction. If what is usually called "the osmotic pressure of a solution" were due to extra bombardment pressure caused by the solute molecules, it would be necessary to apply mechanical pressure from the side of the pure solvent in order to neutralise this pressure. Actually the pressure applied in measuring osmotic pressure is pressure on the solution itself—*i.e.* pressure in the *same direction* as the bombardment pressure of the solute molecules.

This latter objection is avoided by the theory, represented in the discussion by Mr Bousfield and other contributors, that osmotic pressure is equivalent to deficiency in the bombardment pressure of solvent molecules in the solution, in consequence of their dilution by the solute. When, however, we endeavour to obtain the experimental results in terms of this theory, the first objection (apart from other objections to the details of Mr Bousfield's theory) applies with the same cogency as to van 't Hoff's theory. The bombardment pressure inside a collection of molecules of the concentration of a liquid amounts to thousands of atmospheres, and the diminution in pressure owing to dilution of the solvent would be enormously greater than the actual osmotic pressure.

It is not necessary to follow at this point the details of the more purely mathematical discussions as to osmotic pressure. To understand the phenomena it is certainly necessary to return first to the fundamental gas laws and the kinetic theory of gases, and consider what must happen when real molecules are present at high concentrations such as exist in liquids.

According to Boyle's and Charles's laws the volume occupied by a given mass of any gas varies inversely as the pressure and directly as the absolute temperature. This is expressed in the equation $PV = rMT$, where P = pressure in atmospheres, V = volume in litres, M = mass in grammes, T = absolute temperature, and r = a constant which varies for the same mass of different gases[1]. When we embody Avogadro's law in the equation it becomes $PV = \frac{22\cdot 4\,\frac{M}{W}\,T}{273}$ where W = molecular weight of the gas, and $22\cdot 4$ = volume in litres occupied at 0° C. and 1 atmosphere pressure by the number of grammes of a gas which corresponds to its molecular weight. As $\frac{M}{W}$ = the relative number (n) of molecules (or the number of gramme-molecules) present in different masses of different gases, we can write the equation as $PV = \frac{22{\cdot}4nT}{273}$, or, more simply, $PV = {\cdot}082nT$. When n is constant and equal to 1, ·082 is of course the R of the familiar equation $PV = RT$, when P and V are expressed in the units specified. The kinetic theory of gases has made it possible to deduce this equation from simple principles, and also enables us to realise some of its limitations.

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[1] The particular units in which volume, mass, and pressure are expressed have been selected on the score of practical convenience.

The equation represents very satisfactorily the experimentally ascertained facts in the case of permanent gases or mixtures of them within common limits of pressure and temperature, but, as is well known, breaks down outside these limits, and cannot be extended, except in a very limited sense, to vapours, nor in any clearly intelligible sense, to liquids. The causes of these limitations must now be examined in connection with a proposed re-statement of the equation, so as to permit of its extension to vapours and liquids.

In the first place the molecules of the gas, or gas-mixture, may combine with one another chemically, or may dissociate, on altering the temperature or pressure. It is at once evident that the ordinary gas laws cannot be applied to a change of this character, although they apply, just as before, to the products of the change.

The second limitation of the equation becomes evident even at ordinary temperatures and pressures with gases consisting of large molecules, and at high pressures or low temperatures with all the permanent gases, as, for instance, hydrogen. With T and n constant the product PV sooner or later increases at a growing rate with increase of P; and with P and n constant the quotient $\frac{V}{T}$ increases similarly with diminution of T. This is because, although, on an average, each molecule has an amount of free kinetic energy proportional to its absolute temperature, and therefore keeps clear at each temperature an average intermolecular space inversely proportional to the pressure, the space actually occupied by the molecules themselves in virtue of their relative impenetrability and incompressibility becomes an increasing fraction of the total space occupied by the gas. The volume at absolute zero cannot, therefore, be nothing, as the equation would imply; and with increasing pressure at constant temperature the specific gravity of the gas must increase less and less in proportion to the increase of pressure.

It is clear that the equation could only hold good for an ideal gas, the molecules of which were points possessed of mass, but having no extension. Ideal molecules of this kind have, of course, been supposed to exist; but, like many other scientific ideals, they do not correspond with actual experience, the supposition that they exist being, for instance, completely at variance with the results of the experiments of Amagat and others on gases at high pressures.

We have thus reached a first point indicating the necessity of re-stating the gas-laws and the fundamental equation which embodies them. This re-statement, as will appear below, is essential for the application of the gas-laws to liquids. We must distinguish the intermolecular volume, or space kept clear by gas-molecules in virtue only of their movements, from the space they would still occupy if they had no movement capable of external transmission. If we call the latter space, in litres, v,[1] we can then, in accordance with the kinetic theory, write the equation of a gas as $P\,(V - v) = \cdot 082nT$.

When V is constant and $= 1$ litre, v is simply the proportion of occupied space and n is the relative number of molecules or separate particles in a litre, or the molecular concentration. Hence the equation for a gas becomes $P\,(1 - v) = \cdot 082nT$. But $1 - v$ varies as n at any assigned value of P or of T, either in a pure gas or in any particular gas-mixture. Hence if we call N the molecular concentration or number of gramme-molecules in one litre at 0° and a given pressure, the equation $P\,(1 - v) = \cdot 082NT$ will hold at all temperatures with the same pressure and at all pressures with the same temperature. This form of the general equation is of special convenience in connection with the extension of the gas laws to gases at very high pressure and liquids, and will therefore henceforth be used extensively in this paper, although, as will appear below, with an altered definition of P.

It is only at low pressures, where $1 - v$ is practically equal to 1 that P will vary as N when T is constant. Hence Boyle's law could only be practically true at low pressures and fairly high temperatures, and must become less and less true as the pressure rises or temperature falls. Boyle's law must also deviate more, or less, from truth according to the varying size of the molecule in different gases, and consequent varying value of v at the same pressure and temperature. Similarly, it is only when $1 - v$ is practically equal to 1 that with P constant N could vary inversely as T in accordance with Charles's law. Thus at a very high pressure equal volumes of different gases must contain very unequal numbers of molecules, as the experimental results show, and must also expand unequally with the same increments of absolute temperature.

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[1] It should be noted that v represents the volume occupied by the molecules when touching one another, and that this is more than the volume of the molecules themselves. In general v corresponds to the b of van der Waals's equation, but since, in the case of each individual gas, the value selected for the b of this equation depends on the value of the $\frac{a}{v^2}$, I have not adopted van der Waals's notation. It will be shown in a further paper that van der Waals's equation is defective.

Let us now consider the case of two or more gases present together at very high pressure P or low temperature. Let N_1 be the relative number of molecules and $1 - v_1$ the proportion of free space, or intermolecular volume, in a litre of a gas-mixture at 0° at very high pressure, n the number for a given gas in the mixture, N the number and $1 - v$ the intermolecular volume for the same gas in the pure state at the same pressure, and p its partial pressure in the mixture. It is evident that if the gas were present in the pure state the partial pressure p produced by n molecules would be to the total pressure P as n to N. If, now, without altering the pressure or temperature, we substituted for $N - n$ molecules of the pure gas an equal volume of the other gas or mixture of gases, there could be no change in the partial pressure exercised by the n molecules. But the number of molecules in the gas substituted would, as we have just seen, be different from the number displaced, since at the same temperature and pressure v differs for different gases, so that at very high pressures or low temperatures $1 - v_1$ differs correspondingly from $1 - v$. Hence p is to P, not as n to N_1, but as n to N. This is a matter of great importance, as will appear below, in connection with the application of the gas laws to liquids.

Boyle's law could now be re-stated in the wider and more correct, though less convenient, form that the intermolecular volume of a gas varies inversely as the pressure when the temperature is constant. Charles's law would take the form that at constant pressure the intermolecular volume of a gas varies directly as the absolute temperature. For Avogadro's law the re-statement would be that the number of molecules in a given volume of any gas at the same pressure and temperature varies as the intermolecular volume.

As thus re-stated, and with the proviso that no chemical change occurs in the molecules, the gas laws would represent the experimental facts if it were the case that the molecules simply repelled one another on contact. One can imagine an ideal gas with molecules of this kind; and at ordinary temperatures hydrogen seems to behave in practically the same way as this ideal gas. Actually, however, it is clear from the experimental facts that the molecules, when close together, are powerfully attracted to one another, so that, once more, the ideal fails to correspond with the reality. This brings us to a third limitation, involving an additional re-statement of the gas laws, but at the same time allowing them to be extended to liquids.

The mutual attractions of the molecules of all gases are clearly shown by the fact that below a certain critical temperature, and with the assistance, if necessary, of a certain increase of pressure, all gases condense to the liquid form. If the gas laws as just restated were correct the formation of liquids and solids would be totally unintelligible, and substances would always behave as they do when high above their critical temperatures. But even when the molecules are, on an average, so far apart that there is no evident condensation, it must happen that the free kinetic energy of a certain proportion of them is for the moment so small that these molecules, in virtue of their mutual attraction, become associated or aggregated together for the moment in a manner analogous to the mutual association of comets or planets in a solar system, though with the molecules constantly colliding with, and not merely revolving round one another. With diminishing temperature or increasing pressure this must tend to happen more and more, and must thus cause a greater and greater deviation from the simple gas laws, even in their amended form: the effect being to diminish the external pressure which would exist but for the molecules becoming associated. When liquid is formed the great majority of the molecules are associated, so that the external pressure is very small.

Although with increasing association together of the molecules of a gas the external pressure exercised by it becomes less than it otherwise would be, yet the mean intermolecular pressure due to the mutual bombardment of the molecules, must remain the same as if no association had occurred, since the temperature, and consequently the mean kinetic energy of the molecules, is the same. Hence the fundamental gas equation still applies if we take P to mean, not external pressure, but the mean intermolecular pressure. When the gas begins to condense to visible liquid (or even, as will be shown later, to solidify) the equation will still hold good. The mean intermolecular pressure is then the "intrinsic" pressure of the liquid plus its vapour pressure, plus any additional external pressure. At lower temperatures the vapour pressure becomes so small in relation to the intrinsic pressure as to be practically negligible.

We must thus again re-state the gas laws by substituting "intermolecular pressure" for "pressure." In the fundamental gas equation $P(1 - v) = \cdot 082NT$ P now stands for intermolecular pressure, and v for the virtual volume of the molecules in a litre of gas or liquid. We are thus in a position to extend the gas laws to liquids.

In accordance with the molecular conception of matter a liquid is regarded as a system of molecules held together by their mutual attraction, but free to move about within the system except in so far as they collide with one another. They are also kept in constant and very rapid motion by heat. The proportion of free space $(1 - v)$ between the molecules in a litre of the liquid depends on a balance between the attraction of the molecules for one another and the mean free kinetic energy of the molecules. To apply the reasoning on which the kinetic theory of gases is based, this energy will vary, just as with gases, as the absolute temperature T, and will on an average be the same for any sort of molecule or other separate particle present. But since the attractions for one another of the molecules of different liquids vary, the intermolecular pressure, and consequently the free space between the molecules, will also vary in different liquids. The value of $1 - v$ in the fundamental equation is also no longer practically equal to 1, as in gases at ordinary pressures, but is much less than 1. The molecules themselves are also of unequal sizes. Hence equal volumes of different liquids contain very unequal numbers of molecules, and respond with very unequal changes in volume to changes of temperature and pressure. Van der Waals, with the help of his well-known equation, has calculated the value of P for water as about 10,500 atmospheres. If this estimate were correct, and no chemical combination existed among the molecules, the value of $1 - v$ for water would be about 0·118.

When we take P as signifying the intermolecular pressure, and N as the relative number of molecules per litre at 0° and the existing intermolecular pressure of any liquid, we can apply the same equation to liquids as to gases, or to systems of liquid and vapour or gas. If none of the molecules were associated together the external pressure (corresponding to vapour pressure) would be the same as the intermolecular pressure, and the gas would be an ideal or perfect one in the final sense reached in this paper. At temperatures sufficiently above their critical temperatures all the permanent gases approximate to this ideal gaseous state, so that until P becomes so high that $1 - v$ is sensibly less than 1, the gas laws in their crude original form correspond approximately with actual experience. If all the molecules were completely associated together in the liquid form there would be no vapour pressure. At temperatures not far above the melting point but far below the critical temperature this state is approximately realised.

At first sight it might seem that a re-statement of the gas laws in the wider form now given to them is a purely academic procedure, and deprives them of their practical utility, since there are two unknown terms in the re-stated equation, neither of which can be directly measured, though their product is known. It will be seen below, however, that the product of the two unknown terms may be used without either of them being known separately, and that for this reason the equation, in spite of the limitation just referred to, embodies a working hypothesis with very wide and important applications.

2. *Diffusion Pressure and Osmotic Pressure.*

We can now consider the theory of osmotic pressure tentatively suggested by van 't Hoff and accepted by many other writers. This theory has been generally felt, both by chemists and physiologists, to be unsatisfactory, but has become enveloped in a cloud of mathematical reasoning which seems to have hitherto protected it to a considerable extent against attack. Van 't Hoff suggested that the osmotic pressure of a liquid is pressure directly due to bombardment by the solute molecules[1]; and he made the brilliant discovery that for dilute solutions the osmotic pressure corresponds to the external pressure which would be produced if the solute were present as a gas at the existing temperature and in the volume occupied by the solution, with nothing else, or only a gas at ordinary pressure, already occupying this volume.

Now when the solvent (for instance water) is present, the volume is already, as we have just seen, for the most part occupied, and the free space available for the solute molecules is only a small fraction of the total volume. There is thus no intelligible justification for the idea that the addition of the solute molecules would, if they were present under the same conditions as in a gas, add to the existing enormous intermolecular pressure a pressure equal only to what they would produce if the whole space were unoccupied, and they themselves occupied no space. Their actual pressure would be far greater. But even if, in some manner at present quite unintelligible, the intermolecular pressure were increased by an amount just equal to the osmotic pressure, this would give no explanation at all of why pure water should pass *inwards* into the solution through a semi-permeable membrane.

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[1] On this subject van 't Hoff (quoted by Lewis [1916]) wrote as follows: "Regarding osmotic pressure as due to a kinetic cause (*i.e.* as produced by impacts of the dissolved molecules [upon the inside of the semi-permeable membrane]), there must exist a proportionality between the number of impacts in unit time and the number of molecules in unit volume. The proof is therefore exactly the same as that for Boyle's Law."

If a gas osmometer were furnished with a membrane permeable to hydrogen, but not to nitrogen, and if there were first of all pure hydrogen at atmospheric pressure both inside and outside the osmometer, and a little nitrogen were then forced in without altering the volume of gas inside the osmometer, the pressure inside would be increased, but there would be no passage of hydrogen inwards, as the partial pressure of the hydrogen inside would not be diminished. It would be just the same in the case of forcing into water a solute to which an ordinary semi-permeable membrane was impermeable. But if the *volume* of gas in the osmometer were increased, even very slightly, at the same time as the nitrogen was added, hydrogen would immediately begin to diffuse inwards, since the partial pressure of the hydrogen in the osmometer would be diminished in proportion to the increase in volume; and inward pressure proportional to this decrease of partial pressure would be developed.

Now when a solute goes into simple solution (without chemical change, such as hydrate formation) the volume of the liquid is always increased, so that the concentration of the solvent per unit volume is diminished. Just as in the case of the gas osmometer, it is this diminution in the concentration of the solvent that matters as regards osmotic pressure. Van 't Hoff's theory of osmotic pressure concentrated attention on the solute molecules, and not on the dilution of the solvent; and this is one reason for the failure of the theory to explain osmotic pressure.

The molecules of a gas are, apart from the almost negligible restraining influence of gravitation, free to move in all directions except in so far as they are stopped or diverted by collisions with other molecules. Now as all the molecules are in free movement, both of translation and rotation, the chances, whatever they may be, of any one molecule in travelling a definite distance in one direction undergoing a collision which will destroy, or partially destroy, its momentum in that direction, must vary as the proportion of occupied space to total available space, *i.e.* in proportion to v. The number of those which escape, or partially escape, will therefore vary as $1 - v$. The number of molecules of any one kind, which, for a given area of surface and unit of time, travel across this distance, must also vary in proportion to the rate of molecular bombardment of the area—*i.e.* in proportion to the total pressure P if only one kind of molecule is present, or to the partial pressure p of the kind in question if other kinds are present. Hence the rate at which molecules of any one kind penetrate through a given layer of the gas must vary as $P(1 - v)$ or $p(1 - v)$. The rate will of course depend also on the size of the molecules and their mean velocity: also, when the diffusion is through a membrane, on the permeability of the membrane. But when we are only considering relative rates of penetration by the molecules of one particular gas these factors need not be considered, as they are constant. To put this conclusion into other language, *the diffusion rate of any particular gas through a layer of the gas of given surface area and thickness will vary as* $P(1-v)$ *or* $p(1-v)$.

In a gas at ordinary pressure the value of v is so small that it can be neglected where we are comparing variations of diffusion rates with variations of P. Hence in this case diffusion is simply proportional to the pressure P, or for each separate gas to its partial pressure p, where there is a mixture of gases. But in a gas at high pressure the value of v becomes a very appreciable fraction of unity, so that $P(1-v)$ will differ considerably from P. In a liquid such as water the value of $1-v$, as we have already seen, falls to a small fraction of unity. Hence the diffusion rate of water is proportional to only a small fraction of its intermolecular pressure.

We may now consider the case of a gas osmometer with hydrogen at equal but extremely high pressure P inside and outside the membrane, and containing N molecules per litre at 0°. If only pure hydrogen is present on both sides of the membrane the rate of diffusion of hydrogen both inwards and outwards through the molecular layer adjacent to the internal surface of the membrane will be proportional to $P(1-v)$, as we have just seen. But let us suppose that without altering the pressure a mixture containing n molecules of nitrogen to N_1 molecules of hydrogen and nitrogen per litre is substituted for the pure hydrogen in the osmometer and that the mixture is in the perfect gaseous state. In the mixture the value of $1-v$ will be altered to $1-v_1$ and since $\frac{P(1-v)}{P(1-v_1)}=\frac{\cdot 082NT}{\cdot 082N_1T}$, $1-v_1=\frac{N_1}{N}(1-v)$. Hence the rate of diffusion of hydrogen inwards through the surface layer of molecules inside the osmometer will now be proportional to $\frac{N_1}{N}P(1-v)$ and since, as already pointed out, the partial pressure of any gas is proportional to the ratio of its actual concentration to its concentration in the pure state under the same pressure, the outward rate will be proportional to

$$\frac{N_1-n}{N}P(1-v)=\frac{N_1}{N}P(1-v)-\frac{n}{N}P(1-v).$$

Hence the inward rate is in excess in proportion to $\frac{n}{N}P(1-v)$, and as a direct consequence the inward diffusion pressure is in excess in proportion to $\frac{n}{N}P(1-v)$, since at equal temperatures, in accordance with the kinetic theory of gases, the pressure in a given direction varies as the number of molecules passing in this direction in unit of time.

But $\frac{n}{N} P(1-v) = \frac{n}{N} \times \cdot 082NT = \cdot 082nT$. In other words the excess of diffusion pressure inwards owing to the dilution of the hydrogen with nitrogen in the osmometer varies as the outward pressure which the nitrogen would exercise if the molecules occupied no space, so that PV was constant. It is as if PV were constant, whereas it is $P(1-v)$ that is constant. If the molecules were mere mathematical points repelling one another, so that PV was constant and Avogadro's and Dalton's laws held at all pressures the result would be the same with the same molecular concentration, since intermolecular pressure and diffusion pressure would then be identical. For instance, since PV would be constant and $= \cdot 082\ NT$, P for a gas compressed to the same molecular concentration as water at 0°, would be $\cdot 082 \times 55{\cdot}5 \times 273 = 1243$ atmospheres, instead of van der Waals's calculated value of about 10,500 atmospheres; and $\frac{n}{N} P$ would have the value which $\frac{n}{N} P(1-v)$ has.

Now the ratio of excess of diffusion pressure inwards corresponds exactly in amount to what van 't Hoff showed to be the osmotic pressure of a very dilute solution; and this points clearly towards the conclusion that osmotic pressure is closely connected with excess of diffusion pressure of the solvent inwards over that outwards, and that the intermolecular pressure is the same in the solution as in the pure solvent, and remains the same with varying temperature, just as in the gas-mixture supposed.

Let us now consider what happens when we equalise the inward and outward diffusion pressure of the hydrogen by raising the pressure of the gas-mixture inside the osmometer, as in the ordinary method of measuring osmotic pressure. Let p_0 be the added pressure needed to equalise the diffusion pressure of hydrogen; and let N_2 be the molecular concentration of the gas-mixture at the increased pressure. The value of $1-v$ in the gas-mixture will evidently be reduced to $(1-v) \times \frac{P}{P+p_0}$, and the value of the added pressure of hydrogen, since the ratio $\frac{N_1-n}{N}$ is not altered by compression, will be $\frac{N_1-n}{N} p_0$. Hence the equation representing the equilibrium of diffusion pressure will be

$$\frac{N_1-n}{N} P(1-v) + \frac{N_1-n}{N} p_0 = \frac{N_2}{N} P(1-v) \times \frac{P}{P+p_0}.$$

At the ordinary low pressures of gas-mixtures $1-v$ equals 1, and $N_2 \times \frac{P}{P+p_0}$ equals N_1, in accordance with Boyle's law. Hence, since N cancels out, the equation becomes $(N_1-n)P + (N_1-n)p_0 = N_1P$. On solving this equation we find that $p_0 = \frac{n}{N_1 - n} P$. In other words we can equalise the two diffusion pressures of hydrogen by raising the pressure inside the osmometer by a fraction representing the ratio of nitrogen to hydrogen molecules in the mixture. This represents the familiar law of diffusion pressure or osmotic pressure at ordinary gas pressures.

At the other extreme of very high intermolecular pressures, such as those in liquids, the fraction $\frac{P}{P + p_0}$ differs hardly at all from unity until the value of p_0 becomes considerable. Thus if we accept van der Waals's estimate of P for water as about 10,500 atmospheres the value of $\frac{P}{P + p_0}$ will be about 0·998 when p_0 is 30 atmospheres. If the molecules are appreciably compressible[1] $N_2 \times \frac{P}{P + p_0}$ may still be equal to N_1. But even if we consider them as incompressible we can practically simplify the equation into the form

$$(N_1 - n) P (1 - v) + (N_1 - n) p_0 = N_1 P (1 - v);$$

and from this it follows that $p_0 = \frac{n}{N_1 - n} P (1 - v)$, which, in its turn, is equal to $\frac{n}{N_1 - n} \times \cdot 082 NT$. Even, therefore, though we do not know the exact value of P we can still calculate the osmotic pressure of the gas-mixture from its composition if its pressure is extremely high, and the osmotic pressure is very low in comparison.

It is clear also that even if we did not know the molecular concentration of the hydrogen or other "solvent" gas we could still calculate the osmotic pressure if we knew the mass of "solvent" gas in a given volume of the mixture, as compared with the mass in the same volume, and at the same pressure and at 0°, of the pure "solvent" gas, since any error in estimating N would apply equally to the estimate of $N_1 - n$, and would thus cancel out in the equation.

It follows from the equation that if we took P as signifying osmotic pressure and V as volume of "solvent" gas in the mixture, and if n were constant and equal to 1, PV would be equal to $\cdot 082 T$, or to RT. If V were taken as volume of the mixture we should have an equation which could be expressed in the form $P (V - b) = RT$, since $V - b$ would correspond to $N_1 - n$. Sackur has shown empirically that an equation of the latter form gives the osmotic pressure of sugar solutions; but the b of this equation has evidently nothing to do with the b of van der Waals's equation. If the sugar molecules are hydrates the value of the b is of course increased, as the concentration of free water is diminished.

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[1] This question will be discussed in a later paper.

When n is very small relatively to N, it is evident that $\frac{n}{N_1 - n}$ becomes practically equal to $\frac{n}{N}$. Hence at great dilutions van 't Hoff's equation practically gives the osmotic pressure. But as n increases the value of $\frac{n}{N_1 - n}$ diverges more and more from that of $\frac{n}{N}$. Hence van 't Hoff's equation for osmotic pressure gives a value which is increasingly too low, and particularly so if the molecules of the diluent gas are of large size.

If we substitute sugar for nitrogen in the osmometer, and water for hydrogen, the experimental results for osmotic pressure correspond to the values thus deduced theoretically. With very dilute solutions of sugar the results correspond almost exactly with the van 't Hoff equation; but as the strength of the solution increases the van 't Hoff equation gives results which are increasingly too low. Professor Morse and his fellow workers have shown experimentally in their extremely valuable series of researches [1901–1912] that the observed osmotic pressures increase, not in proportion to n, the molecular concentration of the sugar solution, but far more nearly in proportion to the ratio of sugar molecules to weight of water in the solution. This ratio is just the $\frac{n}{N_1 - n} \times N$ of the equation deduced above[1]. The reasoning on which the equation is based thus enables us to predict the experimental results if we assume that the conditions as regards diffusion in the sugar solution are essentially the same as in the very highly compressed gas mixture.

The experimental results for sucrose and dextrose of Morse and his co-workers show that at 0° the osmotic pressure increases with increase of concentration more rapidly than corresponds with the expression

$$\frac{n}{N_1 - n} \times \cdot 082NT.$$

But as is shown in Table 1, reproduced from one of their latest papers [1912], they found that (substituting ·0816 for ·082)[2] the accordance with the above expression becomes closer and closer as the temperature rises, and that at 80° the accordance is exact, even in a solution containing as much as 342 grammes of cane sugar per 1000 grammes of water, or about 28 grammes per 100 cc. of solution, this being the strongest solution of which they measured the osmotic pressure.

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[1] When $N_1 - n$ is taken as 1000 (the weight in grammes of 1 litre of water at 0°) or when $\frac{n}{N_1 - n}$ is multiplied by N, we obtain Morse's "weight concentration" or n. The n of the present paper corresponds, of course, with van 't Hoff's, and not Morse's n.

[2] Morse took 22·3, instead of 22·4, as the volume in litres occupied by one gramme-molecule of a gas at 0° and 760 mm. pressure. Hence ·0816 is substituted for ·082 in the equation in Table I.

TABLE I. *Osmotic Pressures of Cane Sugar Solutions (Morse and Co-workers).*

Value of $\frac{n}{N_1-n} \times N$, assuming that no hydrates are present. (Weight normal concentration of Morse.)	Ratio of observed osmotic pressure to osmotic pressure calculated from the equation $p_0 = \frac{n}{N_1-n} \times \cdot 0816NT$ on the assumption that no hydrates are present.											
	0°	5°	10°	15°	20°	25°	30°	40°	50°	60°	70°	80
0·1	(1·106)	1·082	1·082	1·082	1·084	1·084	1·000	1·003	1·000	1·000	—	—
0·2	1·061	1·063	1·060	1·061	1·062	1·059	1·020	1·011	1·002	1·001	—	—
0·3	1·061	1·058	1·059	1·061	1·060	1·060	1·031	1·024	1·009	0·999	—	—
0·4	1·060	1·059	1·060	1·059	1·060	1·059	1·040	1·038	1·017	1·000	—	—
0·5	1·069	1·067	1·066	1·068	1·067	1·065	1·050	1·046	1·025	1·006	1·000	—
0·6	1·077	1·074	1·073	1·073	1·073	1·071	1·060	1·054	1·032	1·015	1·002	—
0·7	1·083	1·084	1·083	1·083	1·084	1·083	1·069	1·059	1·041	1·020	0·999	—
0·8	1·093	1·093	1·092	1·093	1·093	1·093	1·081	1·067	1·049	1·027	1·008	1·001
0·9	1·104	1·102	1·102	1·102	1·103	1·102	1·089	1·076	1·059	1·033	1·015	1·000
1·0	1·115	1·115	1·113	1·115	1·115	1·113	1·101	1·085	1·071	1·044	1·023	1·000

TABLE II. *Osmotic Pressures of Cane Sugar Solutions at 0°.*

Values of $\frac{n}{N_1-n} \times N$, assuming that no hydrates of sugar are present.	Ratio of observed osmotic pressure to osmotic pressure calculated from the equation $p_0 = \frac{n}{N_1-n} \times \cdot 0816NT$ on the assumption that no hydrates of sugar are present.		Calculated values of $\frac{n}{N_1-n} \times N$, assuming that the equation $p_0 = \frac{n}{N_1-n} \times \cdot 0816NT$ is correct. (From Berkeley and Hartley's data.)*
	Berkeley and Hartley	Morse and Co-workers	
·0992	1·008	1·098	·1000
·2978	1·031	1·057	·3070
·6001	1·062	1·075	·6372
·895	1·096	1·099	·9809
·993	1·109	1·110	1·101
2·376	1·278	—	3·037
3·267	1·374	—	4·489
4·126	1·465	—	6·065

* If the values of $\frac{n}{N_1-n} \times N$ are calculated on the assumption that the sugar is present as a pentahydrate the values will be practically the same as in this column, except in the case of the last two values, which will be slightly higher.

Table II embodies the very exact recent determinations of the Earl of Berkeley and E. G. J. Hartley for the osmotic pressures of cane sugar solutions at 0° C. and widely different concentrations, together with the corresponding results of Morse and his co-workers. The table is calculated from Table III in Berkeley and Hartley's paper [1916]. The first column shows the values which $\frac{n}{N_1-n} \times N$ would have if it were the case that no hydrates of sugar were formed, and that all the water in both pure water and sugar solution were present either as simple molecules of H_2O or as the same mixture of simple and more complex molecules of water.

It was suggested by Callendar [1908], who developed a previous suggestion of Poynting, that the reason why a cane-sugar solution at temperatures near 0° does not vary in a proportion nearly equivalent to "weight normal" concentration is that although no sugar hydrates have been isolated, hydrates nevertheless exist in solution. This is undoubtedly the true explanation, as will appear more fully below. On raising the temperature, or increasing the concentration of the sugar solution beyond a certain point, these unstable hydrates would naturally tend to be dissociated. At a temperature of 80° the dissociation seems, from Morse's data, to be complete, so that no sugar hydrates are present.

TABLE III. *Osmotic Pressures of Cane Sugar Solutions at 0°.*

Weight concentration.		Volume concentration.		Osmotic pressure in atmospheres.			
Grammes $C_{12}H_{22}O_{11}$ per 100 g. H_2O	Grammes $C_{12}H_{22}O_{11}+5H_2O$ per 100 g. free H_2O	Grammes $C_{12}H_{22}O_{11}$ per 100 cc.	Gramme-molecules sugar per litre	Actually observed by Berkeley and Hartley	Calculated from the equation $p_0=\frac{n}{N_1-n}\times\cdot082NT$, assuming that the sugar is present as pentahydrate	Calculated from the same equation, assuming that the sugar is not hydrated. (Morse's theory.)	Calculated from the equation $p_0=\cdot082nT$. (Van 't Hoff's theory)
3·393	4·33	3·32	0·097	2·23	2·24	2·22	2·17
10·18	13·21	9·59	0·281	6·85	6·85	6·66	6·29
20·525	27·39	18·26	0·533	14·21	14·17	13·44	11·95
30·61	42·04	25·81	0·754	21·87	21·80	20·04	16·90
33·945	47·07	28·13	0·822	24·55	24·40	22·22	18·41
81·26	130·54	54·24	1·585	67·74	67·66	53·19	35·48
111·73	199·70	66·14	1·933	100·13	103·50	73·14	42·20
141·11	282·97	75·33	2·201	134·84	146·66	92·37	49·31

Table III shows Berkeley and Hartley's values at 0°, as compared with values calculated from the equation $p_0=\frac{n}{N_1-n}\times\cdot082NT$, on the assumption suggested by Callendar, that the sugar is present as the hydrate $C_{12}H_{22}O_{11}+5H_2O$, which of course leaves less free water present, and thus increases the value of $\frac{n}{N_1-n}$. It will be seen that the calculated values agree very closely with those observed, until the concentration is so high that there is much more sucrose hydrate than water in the solution. The observed values then begin to fall below those calculated, just as might be predicted as probable on the hydrate theory. From Morse's data it appears that in solutions of lower concentration the whole of the sugar is present as pentahydrate until the temperature rises above 25°. The table also has columns showing the figures calculated on Morse's principle from the equation

$$p_0=\frac{n}{N_1-n}N\times\cdot082NT$$

on the assumption that the sugar is not hydrated: also the figures calculated according to van 't Hoff's theory of osmotic pressure from the equation $p_0 = \cdot 082nT$. These columns speak for themselves.

At very low concentrations the values obtained by Berkeley and Hartley diverge from those given by Morse and his co-workers for the same temperature, as shown in Table II; and in all the earlier results, which were obtained at temperatures below 25°, the values of the latter observers at very low concentrations present an anomaly which is very evident in Table I. This anomaly is absent in the results of Berkeley and Hartley at 0°; and it seems almost certain that it is due to a hitherto undetected source of error in the earlier experiments of Morse and his pupils. In these experiments a small proportion of ferrocyanide solution was purposely introduced into the sugar solution and the water, the assumption being that the effect of this on the osmotic pressure would cancel out. But the ferrocyanide, which is ionised and hydrated, must have, on the principles already laid down, the practical effect of increasing the osmotic pressure due to the sugar. Hence the osmotic pressure which would be caused by the sugar solution by itself is lower than the observed osmotic pressure, and in proportion more so at low sugar concentrations. It thus seems possible to account, not only for the general results of osmotic pressure determinations with sugar solutions, but also for the anomalies presented by the early results of Morse and his associates at low temperatures, as compared with the more correct results of Berkeley and Hartley, and of Morse and his associates in their later experiments at higher temperatures.

In so far as we have hitherto considered liquids we have considered solutions as if they were the same as mixtures of perfect gases at very high pressures. But the additional fact must be taken into consideration that the attraction for one another of different kinds of molecules is variable. Thus the different kinds of molecules cannot all come equally close to one another, and the mixture or solution is thus not homogeneous as regards its intermolecular spaces. The attraction of solute molecules for solvent molecules might be either less than, equal to, or greater than the mutual attraction of solvent molecules; and similarly the attraction of solute molecules for one another might be less than, equal to, or greater than their attraction for solvent molecules.

Let us consider the first case. In consequence of the attraction of solute molecules for solvent molecules being less than for solvent molecules to one another the mean clear space round each solute molecule will be greater than round a molecule of solvent. But the result of this will be a surface condensation of solvent molecules round each solute molecule, just as occurs at a free surface. There will thus be local surface tension tending to limit the mean clear spaces round the solute molecules; and the surface condensation will at the same time increase the intermolecular pressure and so diminish the free spaces round the solvent molecules in the area of condensation. Neither in ordinary "crystalloid" nor in "colloid" solutions, nor in gross suspensions, can the distribution of solvent in the space between solute molecules be even, unless solvent and solute molecules have equal attractions for one another.

Now the more the intermolecular pressure round the solute molecules is relatively diminished the more will intermolecular pressure round neighbouring water molecules be increased, provided that a sufficient excess of solvent molecules is present. As a consequence the *mean* intermolecular pressure in pure solvent and in the solution will be practically the same, as will also be the mean clear space per molecule. This would also be the case if the solute molecules had a greater attraction for solvent molecules than solvent molecules have for one another, and likewise if solute molecules had a less or greater attraction for one another than solvent molecules. If solute and solvent molecules had all equal attractions for one another both substances would be in the liquid state: they would be miscible in all proportions; and the intermolecular pressure would be the same at all concentrations. Hence, if P and P_1 be the mean intermolecular pressures in pure solvent and in the solution, P_1 will be equal to P; and if $1 - v$ and $1 - v_1$ be the intermolecular volumes in one litre of pure solvent and of solution, $1 - v_1$ will be to $1 - v$ as N_1 to N (N_1 and N being the numbers of gramme-molecules in one litre of solution and of pure solvent at 0° respectively). The molecules in the two liquids as wholes can thus be treated statistically as if they were homogeneous mixtures of gas molecules at the same intermolecular pressure. In other words, *the intermolecular pressure up to high limits of concentration is the same in pure solvent and solutions in it.* All the experimental data for osmotic pressure and vapour pressure seem to correspond to this conclusion, and the best evidence in favour of it is furnished by this correspondence. The same experimental data show that over a wide range of temperature the intermolecular pressure P remains constant; and further direct experimental evidence on this point will be given in a succeeding paper.

Hence, by the same reasoning as was used in the case of a gas osmometer, the difference between the two diffusion pressures is

$$\frac{n}{N} P (1 - v) = \cdot 082 nT.$$

The osmotic pressure is therefore

$$\frac{n}{N_1 - n} P(1 - v) = \frac{n}{N_1 - n} \times \cdot 082NT,$$

as already shown to be the case from the experimental data. *Osmotic pressure is thus nothing but the excess of pressure required to equalise the diffusion pressure of the solvent from solution to pure solvent with that from pure solvent to solution; and this excess varies as the molecular concentration of the pure solvent, the absolute temperature, and the ratio of solute to solvent molecules.*

It appears that neither van 't Hoff nor succeeding investigators reached any clearly intelligible conception of what osmotic pressure is, though van 't Hoff showed that osmotic pressure is dependent in some way on the gas laws. So long, however, as these laws were embodied in the imperfect equation $PV = \cdot 082nT$, or $PV = RT$, instead of $P(1 - v) = \cdot 082NT$, or $P(V - v) = RT$, the real connection could not be established. Osmotic pressure is commonly referred to as if it were pressure added to a solution in virtue of the presence of the solute, whereas it is simply the increased pressure which must be applied to a solution in order to neutralise the excess in the diffusion pressure of the pure solvent over that of the solvent diluted with the solute. This increased pressure is exactly that required to neutralise the effect of the dilution, just as in the case of a gas mixture at an ordinary pressure; and it is in this indirect way that osmotic pressure depends on the concentration of the solute.

It is evident that with increasing concentration of a solution a point must finally be reached at which, if there are unequal attractions among the molecules, the mean intermolecular pressure begins to alter. Let us consider what must happen in a case not complicated by chemical combination between solute and solvent molecules, or by other chemical changes. The free space round each solute molecule will no longer be controlled by the surface tension resulting from a balancing condensation or rarefaction of solvent molecules round the solute molecule. As a consequence the free spaces round neighbouring solute molecules will finally run together and come to be controlled, not by the mutual affinities of the solvent molecules, but by those of the solute molecules. Unless, therefore, the solute molecules begin to form definite secondary compound molecules they will no longer be "held" in solution by the solvent. In other words the point of saturation will be reached, and the solute molecules can now only exist in the form of aggregates of molecules, whether these aggregates are solid, liquid, or gaseous.

A temporarily supersaturated solution can be prepared by cooling (or heating) a saturated solution, or, in the case of a gas in solution, by lowering the external pressure of the gas. In this case the solute separates out, taking either the solid form as a precipitate, the liquid form as an emulsion, or the gaseous form as free aggregates of gas. It is evident also from the foregoing account that when two or more solutes are present together in simple solution each solute must diminish the solubility of the others. Hence it is possible to "salt out" one solute by adding another.

When solute molecules combine with water to form hydrate molecules this of course diminishes the concentration of free water molecules present in the solution. As a consequence the rate of diffusion of water from solution to pure solvent is diminished. But as the value $1 - v_1$ for the solution is correspondingly diminished as compared with $1 - v$, there is an equal diminution in the rate of diffusion from pure solvent to solution. The net result is that the excess of diffusion pressure of the pure solvent is the same as if no hydrate had been present. The osmotic pressure is, however, increased, since the value of $N_1 - n$ in the osmotic pressure equation is diminished as compared with what it would be without hydrate formation.

If, on the other hand, the solute molecules are partly or wholly ionised, it is equally clear that excess of diffusion pressure must be increased in proportion to the ionisation, provided that the membrane is impermeable to the ions. That increase of osmotic pressure, proportional to the ionisation in the case of very dilute solutions, is actually present was pointed out by Arrhenius. It seems probable that the process of ionisation in an aqueous solution, as was suggested by van der Waals, is a tearing apart of solute molecules owing to the affinity of their constituent atoms or atomic groups for water molecules, with formation of unstable hydrate molecules which cannot pass through the semi-permeable membrane. This hypothesis gets rid of the great difficulties associated with the assumption that ions are free atoms or completely unsaturated groups of atoms, and at the same time renders intelligible the influence of ionisation on osmotic pressure, the absence of any considerable absorption of heat in the process of ionisation, the relations between ionisation and dilution of a solution, and the action of water as a catalyst. The hydrate formation will add to the increase of osmotic pressure due to the mere splitting up of solute molecules in ionisation.

In the case of water, and to a less extent in that of other solvents, there is clear evidence that the molecules are to a large extent chemically combined with one another. This is shown, for instance, by the fact that as the temperature falls the contraction of water becomes less and less for each degree of fall, quite out of proportion to the foregoing re-statement of Charles's law, and that below 4° there is an actual expansion. In this respect water presents a striking contrast to such liquids as mercury or alcohol. A similar anomaly appears in the compressibility of water. These anomalies correspond in direction with the expansion of about 10 per cent. on solidification to ice, so that when molecules of water unite with one another their virtual volume is increased.

Water must evidently be regarded as a solution of compound water molecules in simple H_2O molecules. In the solution the mean intermolecular pressure will therefore, in accordance with the principles already laid down, be the same as if no solute were present. But since the compound solute molecules are readily dissociable into simple H_2O molecules, they will be dissociated instantly in proportion to any fall in the diffusion pressure of the simple H_2O molecules owing to their dilution by an ordinary solute. Thus the compound H_2O molecules will reduce the fall which, but for their presence, would have occurred in the diffusion pressure of the H_2O molecules, and this reducing influence will be proportional to the concentration of the compound molecules. In other words, we must, in estimating the reduction of the diffusion pressure of the simple H_2O molecules, calculate, not from the original concentration of simple H_2O molecules, but from the original total concentration of H_2O molecules, combined or not combined with one another. We must therefore calculate from the "weight concentration" in Morse's sense, allowing only for any water which is present in relatively stable combination with the solute molecules. In calculating osmotic pressure we can thus neglect altogether the fact that the molecules of solvent are to a large extent combined with one another. Hence if we call M, M_1, m, the masses of pure solvent, solution and solute in one litre at 0°, we can write the osmotic pressure equation as $p_0 = \frac{nM}{M_1 - m} \times \cdot 082T$.

The same considerations are valid also in the case of the osmotic pressure of gases at very high pressures. The molecules of the "solvent" gas may be to a large extent aggregated together, and this of course diminishes the external pressure which the same mass would otherwise exert; but in calculating osmotic pressures we have only to take account of the total concentrations of molecules.

In connection with osmotic pressure much confusion has arisen through failure to distinguish between the conditions which determine the amount of osmotic pressure and those which determine whether or not a membrane is permeable to a solute. This confusion occurs more particularly in connection with the theory of I. Traube as to the cause of osmotic pressure, and its relation to the surface tensions of solutions and the intermolecular pressure. His data show an evident relation between lowering of surface tension of a solution and permeability of membranes by the solute, but none between surface tension of solutions and amount of osmotic pressure. It seems clear that the surface tension of a solution (as distinguished from a pure liquid) has no direct relation to its intermolecular pressure. Traube concluded that osmosis is due to the fact that a liquid of high intermolecular pressure absorbs, *ipso facto*, liquids of lower intermolecular pressure. This theory is inconsistent with the principles embodied in the kinetic theory of fluids.

The theory that osmotic pressure is essentially dependent on the existence of chemical combination between solvent and solute molecules has now almost ceased to be supported. It is perhaps, therefore, scarcely necessary to point out that this theory fails altogether to explain the connection between osmotic pressure and the gas laws. Of course molecular attraction plays an essential part in the explanation, but only in so far as it determines solubility.

3. *Vapour Pressures of Solutions.*

The vapour pressure of a substance is the pressure of its vapour at which inward diffusion balances the outward diffusion consisting in the flying off of molecules from the surface. In accordance with the conception of a liquid as a system of freely moving molecules held in proximity to one another by molecular attraction, and subject to the fundamental gas laws, the escape of molecules from the surface is due to the kinetic energy of a certain proportion of them being so great that they can fly off, thus producing external pressure. This proportion must vary inversely as the excess of kinetic energy required to overcome the molecular attraction which holds them; and this energy, as pointed out by van 't Hoff, will vary as the latent heat of vaporisation. The proportion must also depend on the number of molecules per unit of free surface, and on the absolute temperature, since this determines the mean energy of molecular movement.

Since anywhere near ordinary atmospheric pressure $1 - v$ is practically the same in a gas as 1, at these pressures the rate of escape of molecules from the surface will vary with the available excess of intermolecular over "intrinsic" pressure, there being in this reference no appreciable hindrance from the atmosphere. In accordance with the principles laid down in connection with osmotic pressure the rate of passage of vapour molecules from the atmosphere into the liquid must, other things being equal, vary as the pressure of the vapour molecules and as the value of $1 - v$ for the particular liquid, just as in the case of liquid diffusion. Hence if we call the vapour pressure p, the inward diffusion pressure which balances the outward is $p\,(1 - v)$, and not p. The diffusion pressure of a vapour or gas into a liquid is thus only a fraction of the total vapour or gas pressure, just as the diffusion pressure of one liquid into another is only a fraction of its intermolecular pressure.

If, now, a solvent is diluted by the presence of a solute with no appreciable vapour pressure, and the temperature and intrinsic pressure are unaltered, the rate of passage, and consequently the pressure, of vapour molecules outwards will be diminished in the proportion of $\frac{N_1 - n}{N}$, where n, N, and N_1 denote relative number of molecules per litre of solute, pure solvent, and solution. As already seen, the values of $1 - v$ for the pure solvent, and $1 - v_1$ for the solution vary as N to N_1 so long as the intermolecular pressure is the same in pure solvent and solution. Hence if p be the vapour pressure of the pure solvent the diffusion pressure of the solvent outwards from the liquid will be diminished to

$$\frac{N_1 - n}{N}\, p\,(1 - v) = \frac{N_1}{N}\, p\,(1 - v) - \frac{n}{N}\, p\,(1 - v);$$

and the pressure inwards will be altered to $\frac{N_1}{N}\, p\,(1 - v)$. Hence the diffusion pressure outwards will be less by $\frac{n}{N}\, p\,(1 - v)$ than the previous inward pressure. The new vapour pressure will therefore be lower, and if the diminution of vapour pressure be designated p_d, $\frac{N_1 - n}{N}\, p\,(1 - v)$ must be equal to $\frac{N_1}{N}\,(p - p_d)\,(1 - v)$. From this equation it follows that $\frac{n}{N}\, p = \frac{N_1}{N}\, p_d$, so that $p_d = \frac{n}{N_1}\, p$. In other words, at equal temperatures *the depression of vapour pressure of the solvent is proportional to the ratio between concentration of solute molecules and of total molecules in the solution.*

Now this is the proportion discovered experimentally by Raoult. At great dilutions $\frac{n}{N_1}$ is practically equal to $\frac{n}{N}$, the proportion given in van 't Hoff's equation; but both the above theoretical deduction and the actual experimental results (as will be shown below) indicate that Raoult's equation is the correct one.

By similar reasoning it can be shown that if the vapour pressure of a solute in a given solution is p_1, its vapour pressure in stronger or weaker solutions will vary as $\frac{n}{N_1}$.

It has been shown above that within the limits up to which osmotic pressures have hitherto been measured, the osmotic pressure increases in proportion to $\frac{n}{N_1 - n}$, in accordance with the experimental results. But so long as n is small in relation to N_1, as is the case with all existing measurements of osmotic pressure, the difference between $\frac{n}{N_1}$ and $\frac{n}{N_1 - n}$ is small, so that increase of observed osmotic pressure with increase of concentration of solute must run almost parallel with diminution of vapour pressure.

The same factors, such as combination among solute and solvent molecules (as in hydration), or ionisation, which cause increases in osmotic pressure will tell in the same direction, and, up to moderate concentrations, to nearly the same extent, in diminishing vapour pressure. It is evident, however, that the limiting value of p_d is p: whereas the limiting value of $\frac{n}{N_1 - n}$ is infinity. Hence depression of vapour pressure and increase of osmotic pressure must diverge more and more rapidly as the concentration increases, although up to moderate concentration they run nearly parallel.

The parallelism of depressions of vapour pressure with osmotic pressures of limited amount has been inferred by the well-known statical thermodynamical method of Arrhenius, or the cyclical thermodynamical method of van 't Hoff. But the mathematical deduction, with the help of these methods, of the vapour pressure equation $\frac{p_1 - p}{p_1} = \frac{n}{N}$ is imperfect in the absence of experimental proof that osmotic pressure is proportional to $\frac{n}{N}$; and as a matter of fact this proportionality, in whatever way n and N are defined, does not really exist, as already shown.

The approximate parallelism between depression of vapour pressure and osmotic pressure when the molecular concentration of solute is small as compared with that of solvent is shown by the following table constructed from the data of Berkeley, Hartley, and Burton [1909] for solutions of calcium ferrocyanide. It will be seen, however, that osmotic pressure increases more rapidly, in proportion, than depression of vapour pressure.

TABLE IV. *Depression of Vapour Pressure and Osmotic Pressure of Calcium Ferrocyanide Solutions at 0°.*

Grammes anhydrous calcium ferrocyanide per 100 g. water	Osmotic pressure in atmospheres	Percentage depression of vapour pressure of water	Relative values	
			Osmotic pressure	Depression of vapour pressure
31·388	41·22	3·210	1·000	1·000
39·503	70·84	5·400	1·719	1·682
42·889	87·09	6·557	2·113	2·042
47·219	112·84	8·397	2·737	2·620
49·966	130·66	9·674	3·170	3·014

The case of calcium ferrocyanide is of special interest, since not only is the molecule heavily hydrated ($Ca_2Fe(CN)_2 + 11H_2O$), but, as pointed out in a further paper by Berkeley, Hartley and Stephenson [1909], it is associated in solution, besides being ionised to an extent varying with the concentration. In spite, therefore, of the complexity of the conditions, the expected relation between vapour pressure and osmotic pressure holds good generally, as will be shown in greater detail below (Table V).

When both solvent and solute have a sensible vapour pressure that of the solvent will (within wide limits of concentration) diminish, while that of the solute increases, as $\frac{n}{N_1}$. An interesting special case of vapour pressure is presented by solutions of gases. Here vapour pressure is represented by the partial pressure of the gas in equilibrium with its solution. Most of the common gases are relatively very insoluble in water and other liquids, the attraction of solvent for solute molecules being very small. Hence the value of n is very small in relation to N, so that N, N_1, and $N_1 - n$ are sensibly equal, and it does not matter whether the variation of vapour pressure (or gas pressure) is taken as proportional to $\frac{n}{N_1}$, $\frac{n}{N}$, or $\frac{n}{N_1 - n}$. Henry's law, when put into the older form that the mass of a gas which goes into solution in a given mass of solvent varies directly as the partial pressure of the gas, implies that the vapour pressure of the solute varies as $\frac{n}{N_1 - n}$. The law holds better when stated in the more modern form that concentration of the dissolved gas varies proportionally to concentration of the free gas. This implies that the vapour pressure of the solute varies as $\frac{n}{N}$; and the value of $\frac{n}{N}$ is commonly nearer than that of $\frac{n}{N_1 - n}$ to $\frac{n}{N_1}$. The principles just laid down indicate, however, that concentration of the free gas varies as $\frac{n}{N_1}$, *i.e.* as the ratio of molecular concentration of dissolved gas to total molecular concentration of the solution. This appears, therefore, to be the correct form of Henry's law.

Another interesting special case is that of a vapour in contact with its own pure liquid. Here N_1 does not vary, however much of the vapour condenses. Hence the vapour pressure is constant if the temperature does not vary.

When the solvent liquid contains some other solute which does not enter into chemical combination with the gas, the solubility of the latter will be altered in accordance with Raoult's fundamental law of the relation of vapour pressure to molecular concentration of solutions. Hence most gases are relatively insoluble in strong aqueous solutions of other solutes and particularly those which, like caustic soda or potash, or calcium chloride, are both very soluble and form hydrates. Such cases as that of sulphuric acid are of special interest in this connection. The commoner gases are about equally soluble in pure water and pure sulphuric acid, but relatively insoluble in solutions of the one liquid in the other, as Bohr found. This is clear evidence of the presence of hydrates in the solutions, with a consequent great diminution of molecular concentration, and therefore of solubility of a gas at a given pressure, since the value of $\frac{n}{N_1}$ is increased.

An important physiological application of Raoult's law is in connection with the solubility of gases in liquids, where there is no solute entering into chemical combination with the gas. Gases, where this condition is fulfilled, are considerably less soluble in blood and other physiological liquids than in pure water, since the molecular concentration, especially with large protein molecules, is considerably less than in water. Owing to certain ill-defined but very adhesive ideas which have become associated with "colloid" chemistry this matter has been much obscured in recent years.

Other inferences, of fundamental importance both in physiology and physical chemistry, are as follows. The diffusion pressure of a substance from its solution into the pure solvent is, in accordance with the reasoning in the previous section of this paper, equal to $\frac{n}{N} P\,(1-v)$, where n is moles of the substance per litre, N is moles of the pure solvent; and $1-v$ the proportion of free space in the pure solvent. When the diffusion pressure of the substance is balanced by adding to the pure solvent, or to a solution in it, the requisite number (n_2) of moles of the substance, the diffusion pressure of the substance from the original solution is altered to

$$\frac{n}{N} P \times \frac{N_2}{N}(1-v),$$

where N_2 is total moles per litre in the new solution. The diffusion pressure of the new solution into the old is $\frac{n_2}{N} P \times \frac{N_1}{N}(1-v)$, where N_1 is total moles per litre in the old solution. These two values are equal. Hence nN_2 must be equal to n_2N_1, and therefore $\frac{n_2}{N_2}$ must be equal to $\frac{n}{N_1}$. Hence when the ratio of moles of solute to total moles per litre is equal in two solutions in the same solvent they are in diffusion equilibrium for the solute to which the value of n applies. $\frac{n}{N_1} P\,(1-v)$, or $\frac{n}{N_1}\,{\cdot}082NT$ may be designated as the diffusion pressure of the substance. But the further deduction has already been made that the vapour pressure (at not too high a pressure) of a solute varies as $\frac{n}{N_1}$. Hence the vapour pressure of a solute varies as its diffusion pressure when in solution.

Paul Bert was the first to show experimentally that, as has been abundantly confirmed by my own investigations, the physiological action of a gas depends (other things being equal) on its partial pressure. This implies, as already shown, that the action is dependent on the value of $\frac{n}{N_1}$ (n being the molecular concentration of the gas in free solution) in the blood and tissues, and is therefore dependent on the diffusion pressure of the dissolved gas. We have only to substitute "dissolved substance" for "dissolved gas," in order to give Paul Bert's law a far wider scope. It is evidently the variations in *diffusion pressure* of substances within the living organism which matter in a quantitative sense, and to which organic regulation responds. The delicacy with which the partial pressure of CO_2 in the alveolar air, and consequently the diffusion pressure of H_2CO_3 and its ions in the blood, is regulated was shown in 1905 by Priestley and myself in a well-known paper; and in 1912 definite experimental evidence was brought forward by Hasselbalch that it is hydrogen ion concentration to which the respiratory centre responds. It was further shown that there is an almost incredibly delicate regulation of the hydrogen ion concentration (more correctly hydrogen ion diffusion pressure) of the blood [Campbell, Douglas, Haldane and Hobson, 1913].

If we take n as signifying the molecular concentration of free water in an aqueous solution the diffusion pressure of water varies as $\frac{n}{N_1}$, just as does the diffusion pressure of any other substance of which n denotes the molecular concentration. We can then introduce into physiology and physical chemistry the conception of diffusion pressure of water. This is a clear quantitative conception based on the kinetic theory of fluids. The constancy of the depression of freezing-point for blood-serum is well known, and there must of course be a corresponding constancy for osmotic pressure. But depression of freezing-point and osmotic pressure are proportional to $\frac{n}{N_1 - n}$, and not to $\frac{n}{N_1}$, although for low molecular concentrations of a solute the two values are nearly the same. To speak of "the osmotic pressure" of blood and other physiological liquids is clearly, in the light of the conclusions reached above, meaningless except where mechanical pressure is concerned. Experiments recently published by Priestley and myself [1916] on the regulation of excretion of water by the kidneys indicate that diffusion pressure of water in the blood is regulated by the kidneys in a manner comparable as regards extreme delicacy with the regulation of hydrogen ion diffusion pressure.

To demonstrate the existence of blood changes to which the kidneys were re-acting during very rapid excretion of water Priestley had to employ the very delicate electrical conductivity method. This method gave, of course, no direct measure of the diffusion pressure of water in the blood, but in the absence of a sufficiently delicate direct method was very useful for indicating the existence of small changes in the concentration of ionised substances, and thus indirectly of the other substances, which affect the diffusion pressure of water. In this connection it should be noted that for a solution such as blood-plasma or tissue-fluid the value of $\frac{n}{N_1}$ is very different from that of $\frac{n}{N}$, and that for this reason the diffusion pressures of water and other substances are not practically proportional to their concentrations. The molecular concentration (n) of a given substance may differ considerably in different samples of blood-plasma and other physiological fluids, although the value of $\frac{n}{N_1}$ is the same.

4. *Boiling Points of Solutions.*

The connection between the boiling point of a solution and the molecular concentrations of solute and solution follows from the fact that when n gramme-molecules per litre of a solute without sensible vapour pressure are present, the reduction in diffusion pressure outwards of the solvent vapour is $\frac{n}{N} p (1 - v)$. To bring the solution to the boiling point the vapour pressure of the solvent must therefore be raised by the amount (p_r) required to neutralise the deficiency, and so raise the vapour pressure to the boiling pressure p. Hence $\left(\frac{N_1 - n}{N}\right)(p + p_r)(1 - v)$ must equal $\frac{N_1}{N} p (1 - v)$. It follows that p_r must equal $\frac{n}{N_1 - n} p$.

Now although in liquids at the same temperature the total free or externally disposable energy is on an average the same per molecule at each temperature, and varies directly as the absolute temperature, yet the distribution of this energy among the individual molecules is uneven; and in liquids with an appreciable vapour pressure, *i.e.* with part of their molecules in the potentially gaseous state, the potentially gaseous molecules possess more kinetic energy than the liquid molecules. With a given rise of temperature, which of course increases the proportion of potentially gaseous molecules in the liquid, the rise in vapour pressure within the liquid is out of all proportion to the rise in mean intermolecular pressure, or to absolute temperature. If, however, we know the proportional excess of energy required to convert a gramme-molecule of the particular solvent into gas at the temperature concerned we can calculate the elevation of temperature for a given increase of vapour pressure, as van 't Hoff showed. The expression he deduced for the elevation of temperature giving an increase in pressure of $\frac{n}{N}p$ is $\frac{\cdot 002T^2}{H}$, where $H =$ heat of vaporisation per gramme of solvent at its boiling-point. Thus the elevation of boiling point required to give an increase of $\frac{n}{N_1 - n}p$ is

$$\frac{n}{N_1 - n} N \times \frac{\cdot 002T^2}{H}.$$

This corresponds with the experimental results, and the variable factor is not merely n, as in the van 't Hoff expression, but $\frac{n}{N_1 - n} N$, corresponding to Morse's "weight-normal" concentrations. At great dilutions, however, it becomes, of course, practically indifferent which expression is used.

5. *Melting Points.*

When a crystal at its melting point is in equilibrium with the pure solvent from which it has crystallised we must assume that though molecules are constantly flying out from the crystal in consequence of the continuous molecular agitation due to heat, the number of molecules flying out is balanced by the number flying in from the liquid. Of the molecules which fly out we can further distinguish those which penetrate a given small depth of surrounding liquid from those which are stopped and turned back by collisions with other molecules. The former will be proportional to $1 - v$, in accordance with the reasoning already given; and if p denote the total pressure produced by the flying out of the molecules, the outward diffusion pressure produced by those which penetrate the liquid layer will vary as $p(1 - v)$. This will be balanced by a corresponding pressure in the opposite direction. The diffusion pressure will, in fact, vary just in the same way as if the layer of liquid were a completely permeable membrane.

Now let us suppose that without altering the temperature a solute is introduced into the liquid, and that n, N, N_1 denote gramme-molecules per litre of solute, pure solvent, and solution. The diffusion pressure outwards through the liquid layer will, in accordance with the reasoning in the case of osmotic pressure, alter to $\frac{N_1}{N}p(1 - v)$, and that inwards to $\frac{N_1 - n}{N}p(1 - v)$. Hence there will be an excess outwards of $\frac{n}{N}p(1 - v)$, just as in the case of a semi-permeable membrane. The crystal will therefore melt away.

To make the crystal stable again, we must diminish the free energy of its molecules by lowering the temperature, just as in order to neutralise osmotic pressure we must raise the intermolecular pressure of the solution so as to neutralise diffusion outwards. The lowering of temperature will, also, just as in the case of osmotic pressure, be proportional to $\frac{n}{N_1 - n}$. The actual lowering of temperature will, as van 't Hoff showed, depend on the latent heat of solution of the solvent, and is given by the expression $\frac{n}{N_1 - n} N \times \frac{\cdot 002 T^2}{H}$, where H = latent heat of fusion, and T = melting point of pure solvent. This expression is the correct one, and differs from van 't Hoff's through the substitution of $\frac{n}{N_1 - n} N$ for n. When m, M, and M_1 are the masses of solute, pure solvent, and solution per litre, we can substitute $\frac{nM}{M_1 - m}$ for $\frac{n}{N_1 - n} N$, as shown above in the case of osmotic pressure.

When the solute, and not the solvent, crystallises out, the same reasoning applies, and the same equation with regard to alterations of crystallisation temperature with concentration of solute, H being taken as latent heat of solution at original temperature, n as existing concentration of solvent molecules, and N, $N_1 - n$ as original and existing concentrations of solute molecules. Hence if we know its solubility and latent heat of solution at any one temperature we can predict its solubilities at other temperatures, provided that no chemical interaction occurs among solute and solvent molecules.

6. *Experimental Verification of the Theory.*

If the foregoing reasoning with regard to osmotic pressure, diminution of vapour pressure, raising of boiling point, and depression of melting point is correct, it should be possible to deduce from any one of these values any of the others for the same concentration and temperature. Even when the concentration is so high that the intermolecular pressure of the solution has begun to alter it should still be possible to make this deduction, since the proportional effect on all the values will be the same. So long as the intermolecular pressures of solutions remain the same as those of the pure solvents it should also be possible, if the concentrations and specific gravities of the solutions are known, to decide as to the molecular weights of the solutes: in other words to deduce the "molecular" osmotic pressures and depressions of vapour pressure, boiling-point and melting-point. Unfortunately the accurate data at present available for testing the reasoning experimentally are somewhat limited, since comparatively few complete observations have been made on sufficiently concentrated solutions; but the chief of these data may now be considered.

As the relative depression of vapour pressure $\frac{p_d}{p}$ equals $\frac{n}{N_1}$, $\frac{p}{p_d}$ equals $\frac{N_1}{n}$, and $\frac{p}{p_d} - 1$ or $\frac{p - p_d}{p_d}$ equals $\frac{N_1}{n} - 1$ or $\frac{N_1 - n}{n}$. Hence $\frac{p_d}{p - p_d}$ equals $\frac{n}{N_1 - n}$. Hence $\frac{p_d}{p - p_d} \times N$, or N multiplied by the ratio of depression of vapour pressure to vapour pressure of the *solution*, equals $\frac{n}{N_1 - n} \times N$, or gramme-molecules of solute per weight in grammes of one litre of solvent at 0°, and therefore varies as the osmotic pressure, depression of melting-point and rise of boiling-point. Thus we can easily deduce any one of these values from the relative depression of vapour pressure. The molecular osmotic pressure, relative depression of vapour pressure, depression of melting point, and rise of boiling point, are given when the value of $\frac{n}{N_1 - n} \times N$ equals 1. Hence with the molecular fall in the vapour pressure ratio the value of $\frac{p_d}{p - p_d} = \frac{1}{N}$, whereas on the van 't Hoff theory the molecular fall would be given when $\frac{p_d}{p} = \frac{1}{N}$.

Table V shows the osmotic pressures calculated in this way from the relative depression of vapour pressure, as compared with the osmotic pressure actually found by Berkeley, Hartley, and Burton in the experiments already referred to and tabulated in Table IV. For these experiments we have not the data for calculating the molecular weights of the calcium ferrocyanide and its ions.

It will be noticed that the agreement between the calculated values in column 2 and those found is very close, except for the last two observations. In connection with the latter a calculation from the experimental data shows that the observed depressions of vapour pressure must have been appreciably too great; as in spite of the precautions taken the final saturating solution must have increased slightly in concentration, and to an amount which would apparently account for the divergence between calculated and found values. Column 4 shows the increasing divergence between observed values and those calculated on the van 't Hoff theory.

TABLE V. *Depression of Vapour Pressure in relation to Osmotic Pressure.*

Grammes anhydrous $Ca_2Fe(CN)_6$ per 100 g. of total water	Osmotic Pressure		
	Calculated on the theory that $\frac{p_d}{p - p_d} = \frac{n}{N_1 - n}$, and that osmotic pressure $= \frac{n}{N_1 - n} \times \cdot 082NT$	Found	Calculated on the theory that $\frac{p_d}{p} = \frac{n}{N}$, and that osmotic pressure $= \cdot 082NT$. (Van 't Hoff's theory)
31·388	41·21	41·22	39·93
39·503	70·92	70·84	67·18
42·889	87·22	87·09	81·57
47·219	113·88	112·84	104·46
49·966	133·00	130·66	120·34

For aqueous sugar solutions we possess, besides the osmotic pressure observations of Berkeley and Hartley, and of Morse and his co-workers, the determinations by Smits of depressions of vapour pressure, and a series of determinations by various workers of depressions of freezing and elevations of boiling points[1]. As has already been shown, the osmotic pressure results indicate that at 0° cane sugar is present as a pentahydrate in solutions up to high concentrations, but that with rise of temperature the degree of hydration diminishes, and that at 80° the sugar ceases to be hydrated. From similar reasoning it appears that dextrose is present as a di-hydrate at 0°. The following tables show the values calculated for molecular concentrations from the concentrations and experimental results, assuming that the sugar is present in the forms just mentioned. The molecular values are those calculated for a value of 1 of $\frac{n}{N_1 - n} \times N$, or for one gramme-molecule of solute per 1000 grammes of water. The molecular osmotic pressures are divided by T.

Table VI is calculated from the observations by Berkeley and Hartley [1916] of osmotic pressures of cane sugar solution at 0°.

TABLE VI. *Osmotic Pressure of Cane Sugar at* 0°.

Grammes anhydrous sucrose per 100 g. of total water	Gramme-molecules sucrose pentahydrate per 1000 g. free water	Observed osmotic pressure (Berkeley and Hartley)	Molecular osmotic pressure		
			Calculated from data in columns 2 and 3	Calculated from columns 1 and 3 on Morse's principle	Calculated from molecular concentration on Van 't Hoff's principle
3·393	0·1001	2·23	·0817	·0824	·0838
10·18	0·306	6·85	·0820	·0844	·0894
20·525	0·634	14·21	·0820	·0868	·0976
30·61	0·973	21·87	·0824	·0893	·106
33·945	1·089	24·55	·0823	·0909	·109
81·26	3·020	67·74	·0820	·105	·157
111·73	4·621	100·13	·0795	·112	·190
141·11	6·547	134·84	·0755	·120	·230

When the value of $\frac{n}{N_1 - n} \times N$ (or gramme-molecules solute per weight in grammes of one litre of pure solvent) is 1, the value of p_0 $\left(= \frac{n}{N_1 - n} \times \cdot082NT\right)$ is 22·4 when $T = 273$ (0° C.). The molecular osmotic pressure is thus $\frac{22\cdot4}{273} = \cdot082$. It is evident, therefore, from the fourth column of Table VI (with which Table III should be compared) that up to concentrations of about 100 g. anhydrous sucrose per 100 g. of total water the whole of the sugar is present as pentahydrate when the temperature is 0°. At higher concentrations there is evidently a diminishing degree of hydration. It is clear also that consistent figures for molecular osmotic pressure cannot be obtained if the sugar is assumed to be anhydrous (Morse's principle), and that if the molecular osmotic pressure is calculated on van 't Hoff's principle there is no approximation to consistency, except at greater dilutions than the table shows.

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[1] The latter data are collected in Landolt and Börnstein's *Physikalisch-Chemische Tabellen*, 3rd edition.

Table VII is calculated from measurements by different observers of depressions of freezing point, the first seven being quoted by Landolt and Börnstein, while the last four are from observations by Jones and Getman [1901]. This table confirms the conclusions drawn from Table VI. When the value of $\frac{nN}{N_1 - n}$ (molecules solute per 1000 g. water) is 1 the depression of freezing point ought, according to the foregoing reasoning, to be $\frac{\cdot001985T^2}{H}$ and as H (latent heat of fusion of ice) is 79·15, and T is 273, the value of $\frac{\cdot001985T^2}{H}$ (*i.e.* the molecular depression of freezing point) is 1·870. The mean of the values calculated for sucrose pentahydrate from the first eight observations is 1·872. In the case of the last three observations the calculated molecular depression diminishes, just as does the calculated molecular osmotic pressure at similar very high concentrations. The last two columns, which may be compared with the last two columns of Table VI, require no comment.

TABLE VII. *Depressions of Freezing-point of Cane Sugar Solutions.*

			Molecular depression of freezing-point		
Grammes anhydrous sucrose per 100 g. of total water	Moles sucrose pentahydrate per 1000 g. free water	Observed depression of freezing-point	Calculated for sucrose pentahydrate from columns 2 and 3	Calculated for anhydrous sucrose from columns 1 and 3. (Morse's principle)	Calculated directly from molecular concentration. (Van 't Hoff's principle)
·04825	·001409	·00264°	1·874°	1·87°	1·87°
·3414	·00999	·01856°	1·858°	1·86°	1·86°
·6875	·02014	·0378°	1·877°	1·88°	1·89°
2·231	·0656	·1230°	1·875°	1·89°	1·91°
4·465	·1320	·2450°	1·856°	1·88°	1·93°
29·82	·944	1·768°	1·873°	2·03°	2·34°
56·18	1·923	3·630°	1·888°	2·21°	2·98°
68·90	2·461	4·612°	1·874°	2·29°	3·29°
84·46	3·178	5·800°	1·825°	2·35°	3·62°
101·99	4·077	7·230°	1·77°	2·43°	4·02°
122·14	5·27	9·13°	1·72°	2·54°	4·57°

Table VIII is calculated from the observations of Smits on the depression of vapour pressure of cane sugar solution at 0°. It will be seen that the observed depressions agree closely with those calculated on the assumption that the sugar is present as pentahydrate and that the depression (p_d) equals

$\frac{n}{N_1}p$, where p is the vapour pressure of pure water at 0° (4·620 mm. of mercury). The molecular depression ratio calculated from the equation $\frac{p_d}{p-p_d}=\frac{1}{N}$ is for an aqueous solution $\frac{1}{55 \cdot 5}= \cdot 01802$. Column 3 shows that the molecular depression ratios as calculated from the experimental results on the assumption that the sugar is present as pentahydrate correspond with this value. If the depression is calculated on the van 't Hoff theory that $\frac{p_d}{p}=\frac{1}{N}$, or, as already shown in Table IV, on the theory that depression of vapour pressure runs parallel to the osmotic pressure, there is no such agreement.

TABLE VIII. *Depressions of Vapour Pressures of Cane Sugar Solutions at 0°.*

Grammes anhydrous cane sugar per 100 g. total water	Moles sucrose pentahydrate per 1000 g. free water	Observed depression of vapour pressure in mm. of mercury (Smits)	Calculated depression assuming that $p_d=\frac{n}{N_1}p$, and that the sugar is present as pentahydrate	Calculated molecular depression ratio, $\frac{p_dN}{p-p_d}\div\frac{nN}{N_1-n}$
1·711	·0502	·00422*	·00416	·0182
5·896	·175	·01479	·0145	·0183
15·54	·473	·03972	·0391	·0183
34·52	1·104	·09074	·0906	·01806

* In Smits' paper there is an evident misprint (copied in Landolt and Börnstein's *Tabellen*) of 0·00242 for 0·00422.

Table IX, from the observations collected in Landolt and Börnstein's *Tabellen*, gives elevations of boiling point of cane sugar solutions. At temperatures over 80° cane sugar in solution, as pointed out above, is no longer hydrated. Hence the molecular elevation is calculated for anhydrous cane sugar.

TABLE IX. *Elevations of Boiling point of Cane Sugar Solutions.*

Grammes anhydrous sucrose per 100 g. water	Moles sucrose per 1000 g. of water $\left(\frac{nN}{N_1-n}\right)$	Observed elevation of boiling-point. Degrees C.	Molecular elevation (observed elevation divided by $\frac{nN}{N_1-n}$). Degrees C.
2·447	·0715	·035	·49
4·316	·1264	·064	·51
7·25	·212	·103	·49
11·02	·322	·164	·51
16·75	·490	·242	·495
21·68	·634	·317	·50
36·15	1·056	·55	·52
65·47	1·93	1·13	·59
103·4	3·02	2·06	·68
175·1	5·12	3·84	·75
276·2	8·07	6·71	·83

Taking the latent heat of evaporation of water at 100° as 539, the molecular elevation should be $\frac{\cdot 001985 \times 373^2}{5 \cdot 39} = \cdot 512°$. If allowance is made for the increased specific heat of water at 100° this figure is reduced to ·506, which agrees well with the mean of the figures calculated from the observations up to concentrations of 36 g. per 100 g. of water. At higher concentrations, however, the agreement ceases, presumably because the intermolecular pressure of the solution begins to increase.

Table X is for dextrose solutions, and is calculated from Berkeley and Hartley's determinations of osmotic pressure. They give the concentrations in grammes per litre. The concentrations in molecules per 1000 g. of water are calculated from the specific gravity determinations of Tollens, as given by Landolt and Börnstein. It is evident from the table that at 0° the dextrose is present as a di-hydrate, although, just as in the case of cane sugar, there appears to be some dissociation of the di-hydrate in the strongest solution observed.

TABLE X. *Osmotic Pressures of Dextrose Solutions at* 0°.

					Calculated molecular osmotic pressure		
Grammes dextrose per litre of solution	Moles dextrose per litre of solution	Moles anhydrous dextrose per 1000 g. of total water	Moles dextrose di-hydrate per 1000 g. free water	Observed osmotic pressure in atmospheres	For dextrose di-hydrate (columns 4 and 5)	For anhydrous dextrose (columns 3 and 5). Morse's principle	From molecular concentration (columns 2 and 5). Van 't Hoff's principle
99·8	0·554	0·591	0·604	13·21	·080	·082	·087
199·5	1·107	1·265	1·327	29·17	·081	·084	·096
319·2	1·772	2·215	2·407	53·19	·081	·088	·110
448·6	2·491	3·454	3·948	87·87	·082	·090	·129
548·6	3·046	4·669	5·612	121·18	·079	·096	·146

The foregoing tables show close agreement between observed values and those calculated on the new principles deduced in the present paper. The agreement affords strong support to these principles, with the corollary that the kinetic theory of fluids can be applied in a simple and direct manner, not merely to very dilute solutions, but also to strong solutions.

CONCLUSIONS.

Boyle's, Charles's and Avogadro's laws can be more correctly stated, and at the same time extended to liquids, in the form: *the intermolecular volume of a given volume of gas or liquid varies inversely as its mean intermolecular pressure, and directly as the absolute temperature, the concentration of gramme molecules, and a constant which is the same for all gases and liquids.*

This generalisation is an extension of the kinetic theory of gases, and is embodied in the equation $P(V - v) = \frac{22{\cdot}4nT}{273}$, or $P(V - v) = {\cdot}082nT$; where P = mean intermolecular pressure in atmospheres; V = volume in litres; v = volume virtually occupied by the molecules themselves, so that $V - v$ = virtual intermolecular volume; n = relative number of molecules (gramme-molecules); and 22·4 = value of $P\ (V - v)$ when P and $n = 1$ and $T = 273$ (0° C.).

With the help of this equation new quantitative insight is obtained into diffusion pressure, the osmotic pressures, vapour pressures, boiling points, and melting points of solutions, and into other phenomena connected with gases and liquids. It can be shown that:

(1) Diffusion pressure of any one substance between one liquid or gas and another is proportional to the values of its partial pressure p in the one liquid or gas, and $1 - v$ in the other.

(2) The intermolecular pressure is the same for all solutions in the same solvent, up to high concentrations and with wide variations of temperature.

(3) Osmotic pressure is simply the increased intermolecular pressure required to neutralise the excess of diffusion pressure of a pure solvent inwards over that of a diluted solvent outwards, through a membrane permeable to the solvent but not to the solute which dilutes it. This excess is given, up to high concentrations, by the equation $p_0 = \frac{n}{N_1 - n} \cdot 082NT$, where p_0 = osmotic pressure, and n, N, N_1 = gramme-molecules of solute, pure solvent, and solution per litre.

(4) Diminution of vapour-pressure of the solvent and rise of vapour pressure of the solute are proportional to $\frac{n}{N_1}$.

(5) Elevation of boiling point and depression of melting point of the solvent are proportional to $\frac{n}{N_1 - n}$, and can be calculated respectively if the latent heats of boiling or melting of solvent are known.

(6) When the ratio $\frac{n}{N_1}$ is the same in different solutions with the same solvent the substances of which n denotes gramme-molecules per litre (including the solvent) are in diffusion equilibrium, and their mutual diffusion pressure is $\frac{n}{N_1} \cdot 082NT$. This diffusion pressure is of fundamental importance in physiology and physical chemistry, and has often, hitherto, been confused with osmotic pressure.

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XXXVI. STUDIES IN THE ANTITRYPTIC ACTION OF BLOOD SERUM.

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Since Hahn [1897] first definitely observed that the blood serum of normal animals produced an inhibitory action upon the digestive power of trypsin, many investigations have been made into this property, both as regards the nature of the action, and of the particular constituents of the serum which cause it, and as the result several theories have been advanced.

One represents this action as due to the presence in the serum of a true antibody in Ehrlich's sense, analogous to an antitoxin. In support of this numerous statements have been made by Achalme [1901], Jochmann and Kantorowicz [1908], Meyer [1909, 1, 2] and others, that the antitryptic power of the serum of an animal is increased as the consequence of the injection of trypsin into the animal. Meyer [1911] even goes so far as to attribute the presence of antitrypsin in serum to the proteoclastic ferments in the tissue cells acting as antigens, a view which has been reasserted more recently by Stawraky [1914]. The literature however contains much contradictory evidence upon this point, many workers having failed to confirm this increase in antitryptic power after injecting trypsin [Landsteiner, 1907; Doblin, 1909; Rosenthal, 1910, etc.]. Another explanation was given by Rosenthal [1910], who considered that the inhibition could be entirely accounted for by the presence of the products of proteoclastic action. This conclusion, however, was based upon experiments which were not very convincing, and may be dismissed as extremely unlikely; for although the products of a trypsin digest do hinder the action of the enzyme [Bayliss, 1904], yet it is only to a slight extent [Walters, 1912], whilst a very small quantity of serum is capable of producing a very marked inhibition, far too large to be accounted for in this way. Moreover, the antitryptic power of serum is not removed by dialysis, as would be expected if it were due to amino-acids and other products of protein hydrolysis [Meyer, 1909, 1, 2; Cathcart, 1904; Stawraky, 1914].

The view which has been most generally accepted was put forward by [Hedin 1905; 1906, 1, 2; 1907], who attributed the antitryptic action to the adsorption of the enzyme by some constituent of the serum, and pointed out the similarity of the action of serum upon trypsin to the inhibition of tryptic digestion produced by the addition of animal charcoal.

With regard to the nature of the antitryptic substance in the serum, it was shown by Cathcart [1904] that this property was associated with the albumin fraction of the serum, since on fractionating the serum with ammonium sulphate the antitryptic power was retained by the albumin fraction alone, the other serum proteins being quite inactive. Later investigators have attributed the action to lipoids [Schwarz, 1909] on account of the loss of this power when serum is extracted with chloroform. Jobling and Peterson [1914] consider that it is due to the presence of unsaturated fatty acids which may be extracted from the serum by ether or chloroform, by which means they obtained an extract which possessed an actively antitryptic action. They also found that soaps of unsaturated fatty acids obtained from various sources possessed the property of inhibiting antitryptic digestion.

The antitryptic power of serum has been stated to be increased in certain diseases [Brieger and Trebing, 1908; von Bergmann and Meyer, 1908; Hort, 1909; Golla, 1909, etc.], notably in cancer, in certain acute infections such as pneumonia and typhoid fever, in certain chronic infections such as tuberculosis and syphilis, and in severe anaemias, and suggestions have been made to employ this power as a clinical test. This possibility, with the additional hope, no doubt, of a clue to the cancer problem, has inspired a large amount of research in this direction, and methods have been devised by which the antitryptic power of different sera may be compared quantitatively, the comparison with a normal serum being termed the antitryptic index of the serum.

The methods generally used for obtaining this index are the Loeffler plate method, and the Fuld-Gross method. In the former an increasing series of drops of serum are added to a drop of trypsin placed on a surface of coagulated protein, the whole is incubated and the antitryptic index determined from the relative number of drops required just to prevent the trypsin from digesting away and pitting the underlying protein surface. It is obvious that this method is very inaccurate since it is almost impossible to judge whether the surface is indented or not.

In the Fuld-Gross method a definite quantity of caseinogen is employed as substrate, and to this is added such an amount of trypsin as will in a given time digest it to such an extent that the addition of a mixture of alcohol and acetic acid just fails to produce a precipitate. To this mixture of caseinogen and trypsin, a definite amount of serum is added and the antitryptic index is obtained by finding how much more trypsin has to be added to digest the caseinogen to the same degree as before in a given time. This method is not capable of any great accuracy since it depends upon an end point reaction, so that when the quantity of substrate (caseinogen) falls below a certain level the rate of digestion is diminished.

To overcome this Golla [1909] employed a method in which equal ferment strengths were allowed to act on a substrate (caseinogen) to which equal units of normal serum and the serum under examination had been added, and continuous observations were made of the amount of caseinogen digested, either by measuring the increase in electrical conductivity, or the loss in viscosity of the mixtures. This method, however, can only give approximately quantitative results when the two sera under comparison have almost the same action, since Hedin [1906, 1] has shown that small quantities of serum produce a relatively larger effect than large quantities, and therefore when two widely different sera are compared the result will depend upon the quantity of the sera employed for the comparison.

In the course of some experiments upon the antitryptic action of serum it became necessary to compare the action of various sera on trypsin and the unsatisfactory methods usually employed made it desirable to reinvestigate the question of the comparison of two sera using more accurate methods of measuring the antitryptic action than those generally employed.

COMPARISON OF THE ANTITRYPTIC ACTION OF SERA.

Methods employed.

In these experiments equal quantities of a solution of caseinogen (3 per cent. in 0·2 per cent. sodium carbonate) were warmed in a water thermostat at 37° until the temperature of the bath was attained, and the trypsin and serum were then added in the required quantities. After a definite time the quantity of caseinogen digested was estimated by the method used by Hedin [1905] in which a known volume of tannic acid solution was added, the mixture allowed to stand for twelve hours, and the nitrogen determined in a known volume of the filtrate by the Kjeldahl method. The difference between the nitrogen which escapes precipitation with tannic acid before and after the incubation gives a measure of the digestion. As all results are comparative the numbers given in the experiments detailed below represent cc. of $N/10$ acid corresponding to the ammonia distilled off in the Kjeldahl. All the digestions were carried out in the presence of 0·5 cc. of toluene. In each case care was taken to have such an excess of caseinogen present that the action was directly proportional to the amount of active enzyme, whilst the digestions were only extended over short intervals of time, so that the caseinogen available should not fall sufficiently low to affect the rate of hydrolysis. The enzyme solutions employed were a commercial sample made by Fairchild Brothers and Forster, and preparations made by digesting minced ox or sheep pancreas at 37° in the presence of toluene and chloroform for two or three days, filtering, further digesting the filtrate, and dialysing away the products of digestion against running water. These solutions gave only a faint cloudiness with tannic acid, and only a very faint biuret reaction.

Effects of varying the amount of serum.

It was stated by Hedin [1906, 1, 2] that if trypsin and serum were mixed before being added to the substrate, the neutralising effect of the antibody was larger than when they were added separately; moreover the longer the mixture was kept before being added to the substrate, the greater, up to a certain point, was the amount of trypsin neutralised. Thus the adsorption of the enzyme required a certain time to attain its maximum. From this it seemed probable that if trypsin and serum were added together to caseinogen, the rate of digestion would gradually decrease. The following experiment was done to see if such were the case, goat serum being employed.

The mixtures *A*, *B*, and *C* were incubated at 37°, and 50 cc. of each removed at the intervals stated, 30 cc. tannic acid added and the nitrogen estimated in 60 cc. of the filtrate; the increase in nitrogen is given in cc. 0·1 *N* acid.

A. 250 cc. caseinogen, 7·0 cc. water, 2 cc. trypsin (ox pancreas).
B. 250 ,, 6·5 ,, 2 ,, 0·5 cc. serum
C. 250 ,, 6·0 ,, 2 ,, 1·0 ,,

Time hours	Total			Rate per hour during preceding interval		
	A	B	C	A	B	C
1	3·4	2·2	1·6	3·4	2·2	1·6
2	6·4	4·2	2·7	3·0	2·0	1·1
4	12·8	8·0	4·7	3·2	1·9	1·0
8	—	15·6	9·0	—	1·9	1·1

The digestion of the caseinogen proceeded in each case at a rate constant within the error of experiment excepting perhaps for a slight irregularity in the first hour after mixing the solutions.

Under these conditions, therefore, a definite rate of reaction may be obtained.

Hedin found that small quantities of serum had a relatively larger inhibitory effect upon the action of trypsin than large quantities; moreover he observed that no amount of serum was able completely to prevent the action of the trypsin. In the experiments quoted however only a few different concentrations of serum were employed. The following experiments were carried out with a large number of concentrations of two sera of different origin to observe the course of the inhibition in each case, and to ascertain whether a definite quantitative comparison of the two sera could be obtained.

Sheep and dog serum were employed.

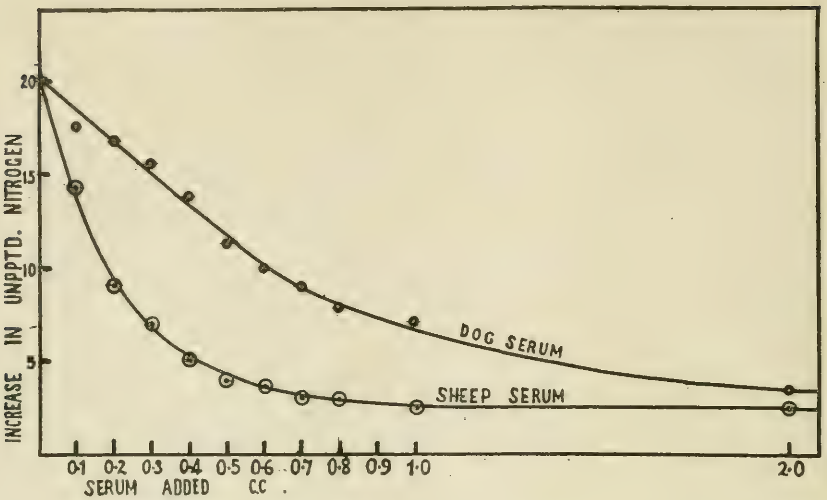
Each mixture consisted of 50 cc. caseinogen solution previously warmed to 37°, 2 cc. trypsin and *N* cc. serum, the total volume being made up to 55 cc. with water. After four hours 30 cc. of tannic acid were added and the nitrogen determined in 70 cc. of the filtrate. The results are given in the following table.

	Serum added in cc.	Soluble nitrogen produced	
		Sheep serum	Dog serum
1	0	20·4	20·4
2	0·1	14·5	17·8
3	0·2	9·1	17·1
4	0·3	7·0	15·6
5	0·4	5·2	13·8
6	0·5	4·1	11·5
7	0·6	3·7	10·0
8	0·7	3·3	9·0
9	0·8	3·2	8·0
10	1·0	2·6	6·1
11	2·0	2·6	3·7
12	3·0	2·7	3·9

1 cc. trypsin in the same volume in absence of serum gave 10·1 cc., showing that the rate in the absence of serum was proportional to the quantity of trypsin present, and that therefore an excess of caseinogen was present in every case.

From these results it is seen that with increasing amounts of serum the relative inhibition produced by each successive addition gradually falls off, until a point is reached beyond which further additions produce no effect and the curve runs parallel to the base line. Complete inhibition cannot be produced.

The figures are shown graphically on the accompanying diagram.



They show further that the sheep serum produced a much greater inhibitory effect than did the dog serum, both in the early stages and in the final rate. It is apparent, however, that a quantitative comparison of the actions of the two sera cannot be obtained by adding a definite amount of serum to a trypsin digest and comparing the action, since the comparison depends upon which part of the curves representing the reaction are used.

A similar experiment was done with another sample of trypsin to try the effect of very small quantities of serum. The sheep serum was diluted ten times with water, and the dog serum with an equal amount of water.

In each case 2 cc. trypsin were employed and 50 cc. caseinogen, the total volume being 55 cc. The mixtures were incubated for four hours at 37°. The figures in the first column give the volumes of undiluted serum contained in the mixtures.

Serum added cc.	Soluble nitrogen produced	
	Sheep serum	Dog serum
0	11·8	11·8
0·02	12·2	—
0·04	11·6	12·5
0·05	—	12·5
0·06	10·3	—
0·08	9·5	—
0·10	8·6	12·3
0·15	6·5	—
0·20	4·9	11·8
0·25	3·6	—
0·4	—	9·2
1·0	—	4·0

1 cc. trypsin in absence of serum gave 5·5 cc.

The experiment shows that with very small quantities of serum very irregular results were obtained. In fact no definite inhibition was observed with the sheep serum until 0·06 cc. had been added, whilst with the dog serum small quantities produced a slight rise. This may be due to the reaction of the serum since a similar slight rise was produced by adding a very dilute mixture of phosphates so that the mixture when diluted to the same volume as the digest gave the same tint with a suitable indicator, as did 0·05 cc. of serum diluted to the same volume.

The action of the same sample of serum upon two different trypsin solutions of the same digestive power.

During the experiments certain discordant results seemed to point to the fact that the different solutions of trypsin were not always inhibited to the same extent by a single specimen of serum, even when the two solutions of trypsin in the absence of serum were capable of digesting the same quantity of caseinogen in the same interval of time. Experiments were carried out therefore upon this point.

I. One sample of trypsin (1) was prepared from ox pancreas, whilst the other (2) was a commercial preparation of Fairchild's trypsin.

The required volumes of the two trypsin solutions which digested the same amount of caseinogen in the same time were determined in preliminary experiments, which also showed that the quantity of caseinogen was such as not to influence the rate of reaction. In each case 50 cc. of caseinogen were employed, the total volume being made to 60 cc. Sheep serum was used and the mixtures incubated at 37° for five hours.

	Digestion with	
Serum added cc.	Trypsin 1 1·45 cc.	Trypsin 2 1 cc.
0	20·0	19·9
0·1	14·1	10·0
0·2	10·3	7·0
0·5	5·0	4·3

II. A similar experiment was carried out with two different samples of trypsin from ox-pancreas and sheep serum—five hours at 37°.

	Digestion with	
Serum added cc.	Trypsin A 2 cc.	Trypsin B 1·6 cc.
0	17·6	17·7
0·1	13·1	11·3
0·2	11·0	7·1
0·5	5·9	4·0

III. A similar experiment with goat serum and two specimens of trypsin (ox pancreas)—three hours at 37°.

Serum added cc.	Trypsin C 4 cc.	Trypsin D 1 cc.
0	12·5	12·6
0·2	8·2	7·6
0·4	5·7	5·7
0·6	4·6	5·0
2·0	3·6	3·5

IV. A similar experiment with goat serum and two more trypsin preparations from ox pancreas—three hours at 37°.

Serum added cc.	Trypsin E 0·7 cc.	Trypsin F 2·6 cc.
0	16·0	16·5
0·2	12·2	12·0
0·4	9·8	10·3
0·6	7·4	8·9
1·0	4·8	7·3
4·0	4·8	7·3

In experiments I and II the two trypsin solutions in the absence of serum digested the same quantity of caseinogen, yet the effect of adding varying quantities of the serum to each was very different. On the other hand in experiment III the effect on the two preparations was practically the same. In experiment IV, again, a considerable difference was noticed as the quantity of serum was increased.

These experiments show, therefore, that equal quantities of a serum may not always produce the same inhibitory effect upon different preparations of trypsin although the enzyme solutions have the same proteoclastic power as measured by the digestion of caseinogen.

In obtaining the antitryptic index of a serum a sample of enzyme is generally employed of such a strength that it will just digest away a given amount of caseinogen in a given time, as in the Fuld-Gross method. As shown above the effect of serum upon this may vary with the particular sample of enzyme used, and it is obvious therefore that the comparison of the antitryptic action of a different serum made at different times with different trypsin preparations, even though of standard strength, may yield erroneous results, and many of the contradictory results found in the literature may probably arise in this way.

From these experiments the conclusion must be drawn that the antitryptic action of the two sera may be compared only by observing the effect of varying quantities of each upon the same sample of trypsin, and that only a qualitative comparison is then possible.

Is this action due to the presence of zymoid in the enzyme preparations?

The different inhibitory powers of the same serum upon different preparations of trypsin might be explained by the presence in the solution of enzyme so altered that it is incapable of digesting caseinogen but is yet able to enter into association with the inhibitory substance in the serum. Evidence of the existence of a modified enzyme which would still combine with antibody but had lost the power of clotting milk, has been put forward by Korschun [1902] in the case of rennet, whilst Bayliss [1904] obtained facts which suggested the production of a similar modification of trypsin by warming to 25° for a day or two. For these modifications Bayliss suggested the name of zymoid. Furthermore Bearn and Cramer [1907] stated that when trypsin was heated to about 50° or 60° the presence of a zymoid was apparent. Experiments were carried out therefore, to see if trypsin could be altered in this manner by heating.

A solution of the trypsin was heated for two hours at 60°, and thus rendered inactive; the addition of this to a mixture of caseinogen, trypsin and serum was without effect upon the quantity of caseinogen digested. Solutions of trypsin were kept at different temperatures, between 30° and 40° for varying times so as to lessen their activity, and quantities of each of similar digestive power to a given volume of the original trypsin, were tested upon caseinogen in the presence of varying quantities of the same serum. In every case it was found that the power of associating with the serum was lost in proportion to the loss of activity. No evidence therefore could be obtained of the presence of zymoid.

Variations in the antitryptic action of serum.

As already stated the antitryptic action of serum has been proposed for diagnostic purposes in disease, a proposal which assumes that the sera of normal animals of the same species have the same inhibitory action upon trypsin. On the other hand it has been contended that considerable variations do occur in the action of normal serum even in the same animal.

In the following experiment the inhibitory action of the serum from three dogs was compared to see if naturally occurring differences could be found. To avoid errors due to different trypsin preparations, the digestions were carried out at the same time using the same trypsin solution. Parallel series of mixtures were incubated for three hours at 37°, and the extent of digestion of the caseinogen measured as before. Each mixture contained 50 cc. caseinogen, 5 cc. trypsin and varying amounts of fresh serum, the total volume being constant. The results are given in the table.

Two of the dogs (1 and 2) were young dogs, about four months of age, and the third was a fully grown female. For comparison also the action of the serum of a goat is given.

Serum added cc.	Soluble nitrogen produced			
	Dog 1	Dog 2	Dog 3	Goat
0	12·6	12·6	12·6	12·6
0·2	10·4	9·6	10·2	8·2
0·4	7·6	7·2	7·6	5·7
1·0	5·1	5·2	5·5	4·0
2·0	4·2	4·2	4·4	3·4
3·0	4·1	4·0	4·2	3·2

The inhibitions produced by the sera from the three dogs were thus substantially the same in every case; the goat serum, however, showed a more marked difference from the others; in an earlier experiment we have already seen that serum from dog and sheep were different in their behaviour.

ATTEMPTED IMMUNISATION AGAINST TRYPSIN.

The question whether the injection of enzyme into an experimental animal is followed by the production of true antibodies has given rise to a considerable amount of work. It has been pointed out by Bayliss [1914, p. 127], that the evidence tends to show that enzymes are not proteins, and as it is very doubtful whether any other substances are capable of acting as antigens and producing antibodies when injected into living organisms, these are *prima facie* grounds for doubting the capacity of enzymes to act as antigens.

The first "anti-enzyme" was described by Hildebrandt [1893], who stated that the injection of emulsin into animals was followed by the appearance in the serum of the power of inhibiting the action of emulsin on glucosides. Bayliss [1912], however, found that this action was due to the diminution of acidity of the mixture, and he showed that when the hydrogen ion concentration was brought back to its original value the effect disappeared, whilst the same inhibitory effect could be produced by making the emulsin to the same hydrogen ion concentration as that produced by adding the immune serum. In the case of the "anti-rennet" produced by immunising animals with rennet, Hedin [1911, 1912] states that it is not a true antibody, whilst Thaysen [1915] says that it may be completely accounted for partly by adsorption of the enzyme, and partly by the effect of change of hydrogen ion concentration.

In the case of trypsin, as stated earlier, many statements have been made to show that an antitrypsin can be produced by the injection of the enzyme, whilst on the other hand there are recorded numerous failures to obtain it. In the case of trypsin the difficulty lies in the presence of the antitryptic property in normal serum. In reviewing the literature it appeared probable that many of the contradictory results might be accounted for by several causes. In many cases the "immune" sera from the experimental animals were tested against the serum of a normal animal of the same species, and it is possible that the antitryptic power of normal animals might vary with the individual. On the other hand, if the blood of the same animal were employed and its antitryptic activity tested before and after injection, the tests would be made usually by the Fuld-Gross method with the fresh serum and thus at different times and possibly with different specimens of trypsin. The latter although of standard activity as regards action on the substrate, might, as has already been shown, be adsorbed to a different degree by the serum, so that it might appear as if the activity of the serum had been altered. In the following experiments attempts have been made to avoid these difficulties by testing the serum of the animal before and after injection against the same trypsin solution. The solutions of trypsin used for injection were rendered as sterile as possible by keeping them for a day or two in the presence of toluene, the latter being then removed by a rapid current of air previously passed through sterile cotton wool. Before the injections were made the experimental animal was bled generally twice with an interval of a week, to obtain two samples of normal serum, which were preserved as described below. The solutions were then injected intravenously, several injections being made at suitable intervals. After a certain time the animal was again bled and the serum collected. In this way two specimens were obtained of the normal serum of the animal for comparison with the serum of the same animal after immunisation, the object being to try their inhibitory effect upon the same sample of trypsin, and to ascertain whether any difference in activity was produced and if so whether this difference was greater than such difference as might exist between the two samples of normal serum. In each experiment the antitryptic action of the serum was tested against the same trypsin preparation as was used for the injection. The sera as they were obtained were dried rapidly by the method recommended by Chapman [1905]. This was done by spreading 10 cc. over the surface of a tared flat glass dish, which was then kept in an evacuated desiccator containing sulphuric acid and placed in an incubator at a temperature of 37°. Within 24 hours the serum dried up. The dish and contents were then weighed and the dried mass powdered and preserved in a dry atmosphere[1] in the ice chest until required, when the powder was dissolved in water and made up to the volume of the original serum from which it had come. The serum when preserved in this manner retains its activity for a long time. In every case the sera were neutralised to litmus paper by the addition of sodium dihydrogen phosphate, before being tested. The tests were made by adding an increasing series of quantities to mixtures of trypsin and caseinogen, incubating at 37°, precipitating with tannic acid, and estimating the nitrogen in the filtrate. The following experiments with the same samples of serum dried and undried show that the drying had not produced any appreciable effect upon the antitryptic activity. The undried serum was kept on ice during the time taken in the drying of the other portion.

I. Each mixture consisted of 25 cc. caseinogen, 1·2 cc. trypsin (sheep pancreas), and varying amounts of the serum, the total volume being made to 30 cc. with water. After 18 hours at 37°, 15 cc. of tannic acid were added to each and the nitrogen estimated in 20 cc. of the filtrate. In the absence of serum 30·2 cc. were obtained.

Serum added cc.	Digestion with	
	Dried serum	Undried serum
0·5	6·0	6·0
1·0	3·6	4·0
2·0	3·1	3·2

II. A similar experiment with 1·2 cc. trypsin (ox pancreas) and sheep serum. In the absence of serum the digestion corresponded to 24·5 cc.

Serum added cc.	Digestion with	
	Dried serum	Undried serum
0·05	22·7	23·1
0·10	21·7	21·3
0·30	14·1	13·9
0·50	10·1	10·1
1·00	6·1	6·4

III. A goat (I) was injected intravenously at weekly intervals with the following quantities of trypsin (commercial sample of Grübler) dissolved in

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[1] On several occasions the serum powder when preserved for some time without special precautions being taken to keep it dry, was found to have become partially insoluble in water. Professor Chapman has informed me that he has noticed the same thing, and he attributes it to the effect of moisture which causes the proteins of the serum to coagulate. This was confirmed by an experiment in which two portions of the same serum powder were preserved, the one in a desiccator containing calcium chloride, and the other in moist air. In the latter case the serum soon became insoluble, whilst in the former it remained perfectly soluble. When precautions were taken to keep the powders dry this insolubility was never observed.

70 cc. of normal saline in each case; 0·4, 0·4, 0·4, 0·6, 1, 1, 1, 1, 1, g. After another week a sample of serum was obtained. Two samples of normal serum were collected before the injections for comparison. The results are given in the following table. Each mixture contained 25 cc. caseinogen, 5 cc. trypsin (1 g. in 50 cc.) and the quantities of serum given below, the total volume being constant.

Serum added	Digestion in the presence of serum		
	Before 1	Before 2	After injection
0·5	17·0	17·4	18·0
1·0	10·1	10·3	10·5

In absence of serum digestion = 28·6.

IV. A goat (II) received five intravenous injections of trypsin solution prepared from ox pancreas, 5, 7·5, 10, 20, 20 cc. being injected at weekly intervals. Two samples of normal and one of "immune" serum were collected.

The action on the enzyme is given below, each mixture containing 2·5 cc. trypsin, 25 cc. caseinogen and varying amounts of the serum. The mixtures were incubated at 37° for 18 hours.

Serum added cc.	Digestion in presence of serum		
	Before 1	Before 2	After injection
0·5	9·1	8·7	9·4
5	2·5	2·4	3·1

In the absence of serum 18·8 cc. were obtained.

V. A goat (III) received three injections intravenously of 20 cc. of trypsin (ox pancreas). Only one sample of normal serum was used. To test the antitryptic activity 2·5 cc. trypsin and 25 cc. caseinogen were digested with the sera, as given below, at 37° for 18 hours.

Serum added cc.	Digestion in the presence of serum	
	Before	After injection
0·01	18·0	18·0
0·05	17·5	17·5
0·10	16·7	16·5
0·25	15·2	14·6
0·50	10·1	9·1
1·00	6·1	5·7

In the absence of serum 18·9 cc. were obtained.

VI. In this experiment a goat was injected intraperitoneally with 20 cc. of trypsin (ox pancreas) on three successive days and was bled after seven days. The injections were not followed by any untoward results in the animal. The mixtures for testing the sera contained 2·5 cc. trypsin, 25 cc. caseinogen and varying amounts of serum, and were kept at 37° for 16 hours.

Serum added cc.	Digestion in the presence of serum		
	Before 1	Before 2	After injection
0·01	18·4	18·1	18·2
0·05	16·8	16·4	17·5
0·10	15·9	15·2	16·3
0·25	10·6	11·0	13·0
0·50	6·5	6·2	6·2
1·00	5·0	4·8	4·5
2·50	4·7	4·6	4·1
5·00	3·9	3·1	3·4

In absence of serum 18·9 cc. were obtained.

Intraperitoneal injection had produced no increased antitryptic action.

The following two experiments were made upon sheep with a sample of trypsin prepared from the pancreas of an animal of the same species.

VII. A sheep (I) was injected intravenously at weekly intervals with 40, 70, 75 cc. of trypsin solution (sheep pancreas). The mixtures for testing the sera containing 1·2 cc. trypsin, 25 cc. caseinogen and varying volumes of serum were incubated at 37° for 18 hours.

Serum added cc.	Digestion in the presence of serum		
	Before 1	Before 2	After injection
0·5	16·2	17·6	16·0
1·0	3·2	3·3	4·0
2·0	2·9	3·1	3·1

In absence of serum—30·2 cc.

The animal then received two further injections of 75 and 175 cc. trypsin.

Serum added cc.	Digestion	
	Before	After injection
0·1	24·8	24·0
0·5	17·7	18·6
1·0	5·9	5·1
2·0	3·0	3·3

In the absence of serum = 29·0 cc.

VIII. A sheep (II) was injected intravenously with a solution of trypsin from sheep pancreas receiving the following doses at weekly intervals—40, 70, 75, 75 cc.

Serum added cc.	Digestion in presence of serum		
	Before 1	Before 2	After injection
0·1	26·5	26·5	26·0
0·2	20·1	24·0	24·4
0·4	19·6	20·5	21·0
1·0	10·3	10·5	11·6
2·0	5·9	6·1	6·3

In absence of serum = 30 cc.

A further injection of 200 cc. of a fresh preparation from sheep pancreas was then given and the serum collected after 14 days and tested against the original normal serum.

Serum added cc.	Digestion	
	Before 1	After injection
0·1	8·3	9·1
0·25	4·7	4·8
0·50	4·5	4·4
1·00	4·5	4·5
1·50	4·2	4·1

In absence of serum = 23·4 cc.

In all these experiments there was no greater difference observed between the antitryptic action of the serum of the animals before and after the injections, than was found between the two samples of serum from the same animal before injection. There was therefore no evidence of the production of any antitrypsin by the injection of the enzyme.

These experiments were extended by examining the sera of the animals to see if the injections had given rise to the formation of a specific precipitin. The serum of goat (I) gave no precipitate when mixed with the trypsin solution used for the injection; on the other hand, after the injection of the trypsin the serum gave a precipitate with dilutions of the trypsin down to 1 in 1000.

The serum of goat (II) injected with ox pancreas trypsin before injection gave no precipitate with the trypsin solution. After injection, however, it gave a distinct precipitate when mixed with the trypsin solution in dilutions down to 1 in 100.

Similarly with goat (III) intraperitoneal injections with trypsin (ox pancreas) produced a faint precipitin reaction.

The sera of the two sheep before injection were also compared with the sera obtained after the final injection of trypsin. On mixing with the solution of trypsin employed, a distinct precipitate was noticed in the presence of 1/100 and 1/1000 respectively of enzyme solution, whereas with the normal sera the mixture remained quite clear.

The last two cases are of interest as an instance of a precipitin being produced by injecting the extract of the pancreas of an animal of the same species.

From these results it is seen that although there was no increased antitryptic power observed in the serum due to the injection, yet a precipitin was produced. They thus show a similarity to the experiments quoted by Bayliss [1912] with emulsin where a precipitin was also produced when enzyme preparations were injected, although he was able to demonstrate that no true antibody to the enzyme was produced at the same time, and a similar conclusion must be drawn here as was done by Bayliss that the antigen to this precipitin was most probably some protein accompanying the trypsin and not the enzyme itself. These experiments contain therefore additional evidence that the enzyme trypsin is not a protein.

The results obtained do not bear out the statement of Porter [1914] that trypsin is inhibited by a specific precipitate. In her case the effect was compared of adding egg white to a mixture of trypsin and serum from a rabbit immunised with egg white, with that produced by adding egg white to a mixture of trypsin and normal serum. The differences shown were exceedingly small however, and no attempt was made to ascertain whether they could not be accounted for by changes in alkalinity of the mixture.

SUMMARY.

The observations herein recorded confirm those of Hedin and show that when increasing amounts of blood serum are added to a mixture of trypsin and excess of caseinogen the inhibitory effect on the trypsin, produced by equal increments of serum, decreases as the total quantity of serum present gets larger, until a point is reached beyond which the further addition of serum produces no further decrease in the action of the enzyme. In no case was it found possible completely to inhibit the action of trypsin by the addition of serum.

Different preparations of trypsin of equal digestive power upon caseinogen are not always inhibited to the same extent by the addition of equal quantities of the same serum.

Experiments to ascertain whether this was due to the presence of a zymoid—*i.e.* altered enzyme which had lost its power of hydrolysing caseinogen but was still able to form an association with the antibody in the serum—were unsuccessful.

The experiments show that in making a comparison of the effect of different sera upon trypsin, it is necessary to employ the same individual preparation of trypsin, and even then only a qualitative result is possible. The so-called antitryptic index, therefore, is not a definite constant for a serum.

In the experiments quoted in this paper the sera of normal animals of the same species did not show any marked variations in "antitryptic action," nor was any great change observed in the serum drawn at different times from the same animal; on the other hand, marked differences were observed between the actions of the serum from different animals (goat, sheep, dog).

It is shown that the possible errors in the methods usually employed for

comparing the effect of sera upon trypsin may account for the contradictory results obtained by so many workers upon the question whether the injection of trypsin into experimental animals is followed by an increase in the antitryptic action of the serum.

Experiments are recorded in which attempts were made to increase the antitryptic action of the serum of an animal by injecting the enzyme intravenously or intraperitoneally, precautions being taken in testing the serum to avoid the errors suggested by the previous experiments.

The action of the "immune" serum in each case was compared with that of two samples of serum drawn from the same animal before the injections were made, and the comparison was made in parallel series of increasing amounts of serum, the same solution of trypsin being employed and the experiments being carried out at the same time. In no case was any increased action observed to follow the injections.

The injections, however, caused the production of a precipitin, which must therefore be attributed to the presence of protein accompanying the enzyme and not to the enzyme itself. These experiments, therefore, bring forward some evidence that trypsin is not a protein.

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